Supporting Tables and Methods

A shortcut to identifying small molecule signals that regulate behavior and development in *Caenorhabditis elegans*

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Supporting Table S1 ^{1}H (600 MHz), ^{13}C (126 MHz), and HMBC NMR spectroscopic data for **ascr#6.1** in methanol-d_4. Chemical shifts were referenced to δ($CD_2\underline{H}OD$) = 3.31 ppm and δ($\underline{C}D_2HOD$) = 49.05 ppm. Coupling constants are given in Hertz [Hz].

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]	^{1}H-^{1}H coupling constants	Relevant HMBC Correlations
1	23.2	1.165	$J_{1,2}$ = 6.2	C-2, C-3
2	67.83	3.73		
3	35.9	1.48		
		1.59		
4	34.2	1.65		
		1.65		
5	72.2	3.79		
6	19.0	1.14	$J_{5,6}$ = 6.1	C-4, C-5
2’	97.1	4.65		C-5, C-3', C-4’, C-6’
3’	69.6	3.72	$J_{2',3'}$ = 1.6, $J_{3',4'ax}$ = 3.1	C-5’
4’	35.6	1.77 (ax)	$J_{4'ax,5'}$ = 11.4, $J_{4'ax,4'eq}$ = 13.1	C-5’, C-6’
		1.95 (eq)	$J_{3',4'eq}$ = 3.6	C-2’, C-3', C-5', C-6’
5’	67.9	3.52	$J_{4'eq,5'}$ = 3.7	C-6’
6’	70.9	3.63	$J_{5',6'}$ = 9.4	C-4’, C-6’
6’-CH_3	17.7	1.21	$J_{6',6'\text{-}CH3}$ = 6.2	C-5’, C-6’

Supporting Table S2 ^{1}H (600 MHz), ^{13}C (126 MHz), and HMBC NMR spectroscopic data for **ascr#6.2** in methanol-d_4. Chemical shifts were referenced to δ($CD_2\underline{H}OD$) = 3.31 ppm and δ($\underline{C}D_2HOD$) = 49.05 ppm. Coupling constants are given in Hertz [Hz].

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]	^{1}H-^{1}H coupling constants	Relevant HMBC Correlations
1	23.2	1.163	$J_{1,2}$ = 6.2	C-2, C-3
2	68.53	3.73		
3	35.9	1.48		
		1.59		
4	34.2	1.65		
		1.65		
5	72.2	3.79		
6	19.0	1.14	$J_{5,6}$ = 6.1	C-4, C-5
2’	97.12	4.65		C-5, C-3’, C-4’, C-6’
3’	69.58	3.72	$J_{2',3'}$ = 1.6, $J_{3',4'ax}$ = 3.1	C-5'
4’	35.60	1.77 (ax)	$J_{4'ax,5'}$ = 11.4, $J_{4'ax,4'eq}$ = 13.1	C-5’, C-6’
		1.95 (eq)	$J_{3',4'eq}$ = 3.6	C-2’, C-3’, C-5’, C-6’
5’	67.94	3.52	$J_{4'eq,5'}$ = 3.7	C-6’
6’	70.87	3.63	$J_{5',6'}$ = 9.4	C-4’, C-6’
6’-CH_3	17.79	1.21	$J_{6',6'\text{-}CH3}$ = 6.2	C-5’, C-6’

Supporting Table S3 ^{1}H (600 MHz), ^{13}C (126 MHz), and HMBC NMR spectroscopic data for **ascr#7** in methanol-d_4. Chemical shifts were referenced to δ($CD_2\underline{H}OD$) = 3.31 ppm and δ($\underline{C}D_2HOD$) = 49.05 ppm. Coupling constants are given in Hertz [Hz].

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]	^{1}H-^{1}H coupling constants	Relevant HMBC Correlations
1	170.1			
2	122.8	5.84	$J_{2,3}$ = 15.3, $J_{2,4}$ = 1.5	C-1, C-4
3	150.8	6.99	$J_{3,4}$ = 6.8	C-1, C-4, C-5
4	29.4	2.40		C-2, C-3, C-5
		2.33		
5	36.8	1.70		C-3, C-4,
		1.65		
6	71.6	3.83		C-4, C-5, C-2’
7	19.2	1.16	$J_{6,7}$ = 6.1	C-5, C-6
2’	97.4	4.66		C-3’,C-4’, C-6’
3’	69.9	3.73	$J_{2',3'}$ = 1.6 $J_{3',4'ax}$ = 3.1	C-5’
4’	36.0	1.78 (ax)	$J_{4'ax,5'}$ = 11.4, $J_{4'ax,4'eq}$ = 13.0	
		1.96 (eq)	$J_{3',4'eq}$ = 3.4	C-3’, C-5’
5’	68.4	3.52	$J_{4'eq,5'}$ = 4.5	
6’	71.3	3.61	$J_{5',6'}$ = 9.4	C-4’
6’-CH_3	18.2	1.22	$J_{6',6'\text{-}CH3}$ = 6.3	C-5’, C-6’

Supporting Table S4 ^{1}H (600 MHz), ^{13}C (126 MHz), and HMBC NMR spectroscopic data for **ascr#8** in methanol-d_4. Chemical shifts were referenced to δ($CD_2\underline{H}OD$) = 3.31 ppm and δ($\underline{C}D_2HOD$) = 49.05 ppm. Coupling constants are given in Hertz [Hz].

Position	δ ^{13}C [ppm]	δ ^{1}H [ppm]	^{1}H-^{1}H coupling constants	Relevant HMBC Correlations
1’	166.4			
2’	125.0	6.17	$J_{2',3'}$ = 15.3, $J_{2',4'}$ = 1.5	C-1’, C-4’
3’	147.2	6.98	$J_{3',4'}$ = 6.8	C-1’, C-4’,C-5’
4’	29.1	2.44		C-2’, C-3’, C-5’
		2.38		
5’	36.8	1.78		C-3’, C-4’, C-2'
		1.69		
6’	71.4	3.86		C-4’, C-5’
7’	19.0	1.18	$J_{6',7'}$ = 6.1	C-5’, C-6’
2’’	97.4	4.68		C-3’’, C-4’’,C-6’’
3’’	68.5	3.74	$J_{2'',3''}$ = 1.6, $J_{3'',4''ax}$ = 3.1	C-5’’
4’’	35.8	1.80 (ax)	$J_{4''ax,5''}$ = 11.2, $J_{4''ax,4''eq}$ = 13.0	
		1.97 (eq)	$J_{3'', 4''eq}$ = 3.6	C-2’’, C-3’’,C-5’’, C-6’’
5’’	68.0	3.51	$J_{4''eq,5''}$ = 3.6	
6’’	71.1	3.63	$J_{5'',6''}$ = 9.4	C-4’’
6’’-CH_3	18.1	1.22	$J_{6'',6''\text{-}CH3}$ = 6.0	C-5’’, C-6’’
1	132.3			
2	131.1	7.63		C-4, C-6, 1-COOH
3	119.8	7.96		C-1, C-5
4	142.4			
5	119.8	7.96		C-1, C-5, 1-COOH
6	131.0	7.63		C-4, C-6
1-COOH	174.4			

Supporting Methods –

1. Differential Analysis by 2D-NMR Spectroscopy. Crude metabolite extract (derived from 500 ml of *daf-22* or N2 liquid culture) was adsorbed on 4 g of Celite and dry loaded into a empty 25 g solid-load sample cartridge and filtered over a RediSep® Normal-Phase Silica Flash Column using a dichloromethane-methanol solvent system, starting with 5 min of 100% dichloromethane, followed by a linear increase of methanol content up to 10% at 10 min, followed by an increase of methanol content to 100% at 25 min. Fractions eluting with up to 50% methanol were combined, evaporated to dryness, and 30-50 mg of the residue was suspended in 0.75 mL of methanol-d_4. The suspension was centrifuged to remove insoluble materials, and the supernatant was subjected to NMR spectroscopic analysis.

The ^{1}H NMR spectra of metabolite extracts derived from different batches of liquid culture frequently revealed considerable variation of proton chemical shift values. For example, the chemical shift of the proton in position 2 of nicotinic acid, a component of the media present in all metabolite extracts, varied between 8.6 and 8.7 ppm. Such variation of chemical shift values between samples must be minimized for successful comparison of 2D NMR spectra via overlays. In order to reduce chemical shift variation between different samples, we carefully adjusted the pH values of all metabolite extracts prior to 2D NMR-spectroscopic analysis. For this purpose, we titrated the metabolite extracts with 5% acetic acid-d_4 in methanol-d_4, using the proton chemical shifts of nicotinic acid as an indicator for change in pH. Chemical shift variability was greatly reduced by using this method, significantly improving the quality of the dqfCOSY overlays. High-resolution dqfCOSY spectra were acquired using the following parameters: acquisition time 0.6 s; 75 increments (ni) per 1 ppm of sweep width. Phase cycling was used for coherence selection, and MestreC was used to process the resulting data, zero-filling the spectra to 8096 complex data points in the directly detected dimension (F2) and 2048 data points in the indirectly detected dimension (F1). Bitmaps derived from absolute-value processed dqfCOSY spectra were then imported into Adobe Photoshop CS3 and overlayed as described in reference [1]. Various combinations of difference and multiply filters were used to highlight components that were distinctly present or absent in the N2 and *daf-22* spectra.

2. Mating assay. The mating assays were performed as previously described[2]. All assays were conducted at 20 ^{o}C and each ascaroside was tested on at least two different days. For dose response curves, each dilution was tested on at least three different days.

3. Dauer formation assay. Standard methods were used to grow worms in liquid cultures, modified as follows[3]. Worms were washed into two 5 cm NGM plate into a 15 ml falcon tube, treated with 5 ml of bleach solution (7 ml dH_2O, 2 ml bleach, 1 ml 5 M KOH), and vortexed for 90 s. Subsequently, the worms were pelleted using a tabletop centrifuge, and the supernatant was removed. Additional 2 ml of bleach solution was added and the tube was vortexed for 5 minutes until all worms had dissolved. The tube was then centrifuged for 1 minute to pellet the eggs. The supernatant was removed and the eggs were washed twice with S-complete medium.

For the dauer assay, eggs were suspended in 2 ml of S-complete medium at a concentration of 1 egg/µl, and *E. coli* (HB101) was added for a final concentration of 0.5 mg/ml. Aqueous solutions of ascarosides were added to achieve two different concentrations (40 nM and 200 nM) and the worms were grown at 20 ^{o}C on the carousel. After 6 days, dauer formation was assessed by soaking the worms in 1% SDS and counting worms that are resistant to SDS[4]. Each compound was tested at least 5 times on 5 different days.

4. Partial purification of ascr#8, ascr#6.1, and ascr#6.2. The following chromatographic fractionations were monitored for the presence of signals the double bond and aromatic spin systems of ascr#8 by using ^{1}H and dqfCOSY NMR spectra.

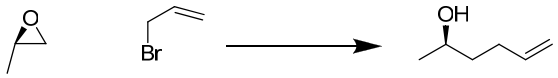
Metabolite extract derived from 20 L of wildtype (N2) liquid culture was fractionated over a 1 kg-reversed-phase (Sigma-Aldrich octadecyl-functionalized silica gel) column, using a gradient solvent system starting with 4% methanol in water containing 1% of acetic acid. The methanol content was increased step-wise up to 100%. The fraction eluting at a methanol content of 10-20% was evaporated to dryness and fractionated further using normal-phase column chromatography on silica, using dichloromethane-methanol mixtures as solvent, starting with 10% methanol, which was increased step-wise to 50%. ascr#8 containing-fractions containing were re-fractionated *via* reversed-phase column chromatography using a 13 g C_{18} RediSep column (Teledyne ISCO) using methanol-water mixtures as solvent, starting with 10% of methanol, which, after 3 min, was increased linearly to 100% over 24 min. One main fraction containing ascarosides ascr#6.1, ascr#6.2, and ascr#8 was obtained, which was characterized by

dqfCOSY, TOCSY, NOESY, and HMBC spectra. Additionally, this mixture was characterized *via* HPLC-MS using a Supelco Discovery HS C-18 column (25 cm x 10 mm, 5 µm particle diameter) and a water-methanol solvent gradient starting with 20% methanol, which was increased to 100% over a period 30 min. ESI-MS (m/z) for ascr#8: $[M+Na]^+$ calcd. for $C_{20}H_{27}NO_7Na$, 416.4; found, 416.4. ESI-MS (m/z) for ascr#6.1 and 6.2: $[M+Na]^+$ calcd. for $C_{12}H_{24}O_5Na$, 271.3; found, 271.3.

5. Identification of long-chained ascarosides in *daf-22* metabolite extracts. Metabolite extract derived from 4 L of *daf-22* liquid culture was chromatographed over silica, using a 7:1 dichloromethane:methanol mixture containing 2% acetic acid as solvent. Fractions were analyzed by ^{1}H and dqfCOSY NMR spectroscopy, and ascaroside-containing fractions were pooled, evaporated *in vacuo* and rechromatographed using dichloromethane:methanol mixtures as solvent, linearly increasing methanol content from 4% to 10%. NMR spectroscopic analysis of fractions eluting at a methanol concentration of 7% revealed the presence of ascarosides, which positive-ion electrospray analysis showed to correspond to a mixture of long-chained derivatives[5]. Major components were 28-(3'*R*,5'*R*-dihydroxy-6'*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy)-2-nonacosanol (positive-ion ESI-MS (m/z): $[M+Na]^+$ calcd. for $C_{35}H_{70}O_5Na$, 593.5; found, 593.5) and 30-(3'*R*,5'*R*-dihydroxy-6'*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy)-2-untriacontanol (positive ion ESI-MS (m/z): $[M+Na]^+$ calcd. for $C_{37}H_{74}O_5Na$, 621.5; found, 621.5). In addition, variable amounts of 5'-O-acetylated derivatives of these long-chained ascarosides occur in both wild-type and *daf-22* extracts [characteristic NMR-spectroscopic data for the acetylated derivatives: ^{1}H NMR (600 MHz, acetone-d_6) 1.10 (d, J = 6.2 Hz, 6'-CH_3), 1.11 (d, J = 6.2 Hz, 3 H, 2'-O-CH-C$\underline{H}_3${side chain}), 1.45 (m, 1 H, 2'-O-CH-C$\underline{H}$H{side chain}), 1.55 (m, 1 H, 2'-O-CH-CH$\underline{H}${side chain}), 1.79 (ddd, J = 13.8, J = 11.5, J = 3.1 Hz, 1 H, 4'-H_{ax}), 2.02 (m, 1 H, 4'-H_{eq}), 3.73 (td, J = 3.1, J = 1.5 Hz, 1 H, 3'-H), 3.77 (m, 1 H, 2'-O-C$\underline{H}${side chain}), 3.81 (dq, J = 9.8, J = 6.3 Hz, 1 H, 6'-H), 4.69 (br. s, 1 H 2'-H), 4.86 (ddd, J = 11.3, J = 9.8, J = 4.8 Hz, 1 H 5'-H); ^{13}C NMR (126 MHz, acetone-d_6) 18.6 (6'-$\underline{C}H_3$), 20.1 (2'-O-CH-$\underline{C}H_3${side chain}), 33.7 (C-3'), 38.6 (2'-O-CH-$\underline{C}H_2${side chain}), 68.3 (C-6'), 69.6 (C-3'), 71.3 (C-5'), 72.5 (2'-O-C{side chain}), 97.9 (C-2'); NMR-spectroscopic signals of the acetyl group; ^{1}H NMR (600 MHz, acetone-d_6) 2.00 (s, 3 H); ^{13}C NMR (126 MHz, acetone-d_6) 21.8, 170.9].

6. Syntheses

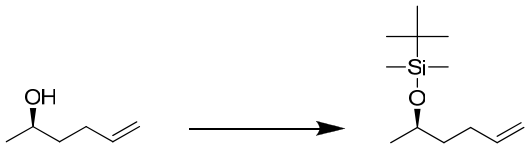
6.1 2*R*-hex-5-enol:



1 2 7

A multi-neck round bottom flask under argon was fitted with a 100-mL dropping funnel was charged with magnesium (5.16 g, 215 mmol), THF (50 ml), and a single crystal of iodine. The addition funnel was charged with allyl bromide (15.72 g, 130 mmol) and THF (50 mL). A 5-mL portion of the allyl bromide solution was added and the Mg-containing suspension was allowed to stir until the brown color disappeared. After waiting an additional ten minutes, the remaining allyl bromide/THF solution was added drop-wise over a 90-minute period. Following completion of addition, the mixture was allowed to stir for an additional hour. The mixture was brought to -40 °C and copper iodide (620 mg, 3.25 mmol) was added. The resulting light green mixture was allowed to stir an additional 30 min, at which time *R*-propylene oxide (2.5 g, 43 mmol) in THF (20 ml) was added in a single portion. The mixture was allowed to warm to -15 °C over another 2 h and to warm to room temperature while stirring for 18 h. Subsequently, the reaction mixture was cooled to 0 °C and saturated aqueous ammonium chloride solution (120 mL) was added. The organic phase was separated, and the aqueous phase was extracted with three 50 mL aliquots of ether. The organic phases were combined and dried over sodium sulfate. After decanting, the solution was concentrated under reduced pressure yielding **7** (3.5 g, 35 mmol, 82% yield) as a colorless oil, which was used in the next step without further purification.

6.2. *O*-(*t*-butyldimethylsilyl)-2*R*-hex-5-enol

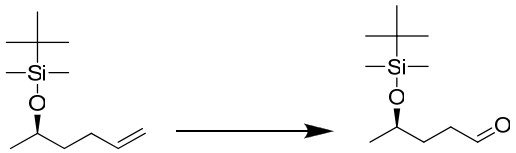


7 8

A 250 mL round-bottom flask under argon was charged with a solution of **7** (3.51 g, 35 mmol) dissolved in dry DMF (60 mL). The mixture was cooled to 0 °C prior to addition of imidazole (5.15 g, 76 mmol) and allowed to equilibrate for ten minutes. *tert*-Butyldimethylsilyl chloride (10.4g, 69 mmol) was added *via* syringe, and the reaction mixture was allowed to stir 24 h while gradually warming to room temperature. Subsequently, aqueous saturated $NaHCO_3$ (200 mL)

was added to the mixture and the resulting biphasic solution was extracted with three 50 mL aliquots of pentane:ether (9:1 *{v/v}*). Organic phases were combined and dried over sodium sulfate. The solution was then decanted and concentrated under reduced pressure before being purified by silica gel column chromatography (1:10 ethyl acetate: hexanes to 1:2 ethyl acetate: hexanes *{v/v}*), yielding **8** (5.3 g, 25 mmol, 71%) as a colorless oil.

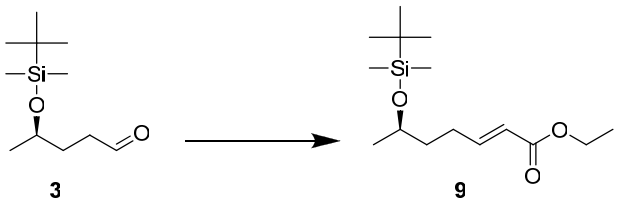
6.3. 4*R*-(*t*-butyldimethylsilyloxy)pentanal



8 **3**

To a solution of **8** (1.83 g, 7.5 mmol) in dry DCM (30 mL) sodium bicarbonate (20 mg) was added to neutralize any acid formation during ozonolysis. The flask was cooled to -78 °C and ozone was bubbled through for four hours at an oxygen flow rate of about 10 ml/min. It was observed that at this temperature, the reaction proceeded extremely slowly. Therefore, the solution was allowed to warm very gradually to room temperature while ozone was continuously administered. After 16 h, dimethyl sulfide (15 g, 250 mmol) was added to the reaction vessel and the resulting solution stirred at room temperature for an additional 48 h. Progress of the reduction of the ozonide was monitored by TLC. Subsequently, the solution was concentrated *in vacuo* and purified by silica gel column chromatography (12:1 hexanes:ethyl acetate {v/v}), yielding 0.93 g of **3** as a colorless, viscous liquid (4.3 mmol, 57%). ^{1}H NMR (600 MHz, acetone-d_6) δ 0.07 (s, 3 H, ($C\underline{H}_3$)$_2$-Si), 0.073 (s, 3 H, ($C\underline{H}_3$)$_2$-Si), 0.89 (s, 9 H, *t*-butyl), 1.14 (d, *J* = 6.1 Hz, 3 H, 5-H), 1.62-1.75 (m, 2 H, 3-H), 2.47-2.51 (m, 2 H, 2-H), 3.91 (m, 1 H, 6-H), 9.745 (t, *J* = 1.6 Hz, 1 H, 1-H).

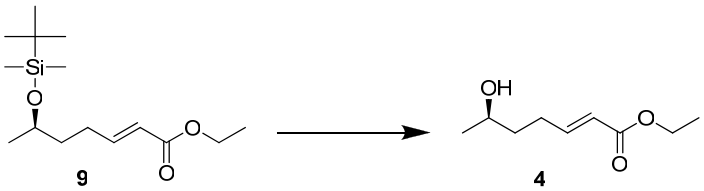
6.4. 6*R*-(*t*-butyldimethylsilyloxy)hept-2*E*-enoic acid ethyl ester



3 9

To a solution of triethyl phosphonoacetate (0.62 g, 2.8 mmol) in trichloroacetonitrile (30 ml) stirred at room temperature, lithium chloride (0.18 g, 4.2 mmol), diisopropylethylamine (0.36 g, 2.8 mmol), and **3** (0.30 g, 1.4 mmol) were added successively and the reaction was stirred overnight. Reaction progress was monitored *via* TLC (12:1 hexanes: ethyl acetate *{v/v}*) and was found to be complete after 42 h of stirring. The solution was concentrated under reduced pressure and purified using silica gel column chromatography (1:8 ethyl acetate:hexanes to1:1 ethyl acetate:hexanes *{v/v}*). The resulting viscous liquid was analyzed by ^{1}H NMR and conclusively shows **9** with a 98:2 ratio favoring the desired *trans* arrangement across the double bond (0.36 g, 1.26 mmol, 90% yield). ^{1}H NMR (500 MHz, acetone-d_6) δ 0.07 (s, 6 H, (C$\underline{H}_3$)$_2$-Si), 0.89 (s, 9 H, *t*-butyl), 1.15 (d, *J* = 6.1 Hz, 3 H, 7-H), 1.23 (t, *J* = 7.2 Hz, 3 H, CH$_2$C$\underline{H}_3$), 1.55-1.60 (m, 2 H, 5-H), 2.21-2.39 (m, 2 H, 4-H), 3.90 (sext., *J* = 6.1 Hz, 1 H, 6-H), 4.12 (q, *J* = 7.1 Hz, 2 H, C$\underline{H}_2$CH$_3$), 5.83 (dt, *J* = 15.6, *J* = 1.6 Hz, 1 H, 2-H), 6.96 (dt, *J* = 15.6, *J* = 7.0 Hz, 1 H, 3-H).

6.5. 6*R*-hydroxyhept-2*E*-enoic acid ethyl ester

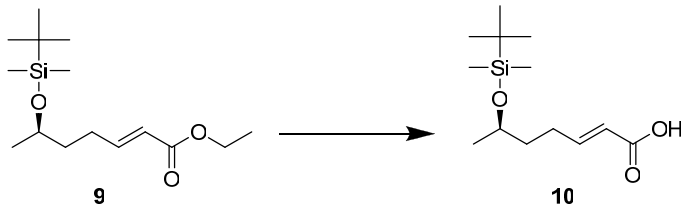


9 4

To a vial containing **9** (0.36 g, 1.25 mmol) dissolved in acetonitrile (3 ml), 40% HF/H$_2$O (0.75 ml) was added drop-wise and the solution was allowed to stir for 1 h. The reaction was judged complete by TLC (1:1 hexanes: ethyl acetate *{v/v}*), and aqueous saturated NaHCO$_3$ solution (3 ml) was added to neutralize the reaction. Desired materials were extracted from the aqueous solution using three 1 ml-aliquots of 1:1 ether: pentanes. Organic layers were combined and dried over sodium sulfate, filtered, and concentrated under reduced pressure before being purified by silica gel column chromatography (1:9 ethyl acetate:hexanes to 1:4 ethyl

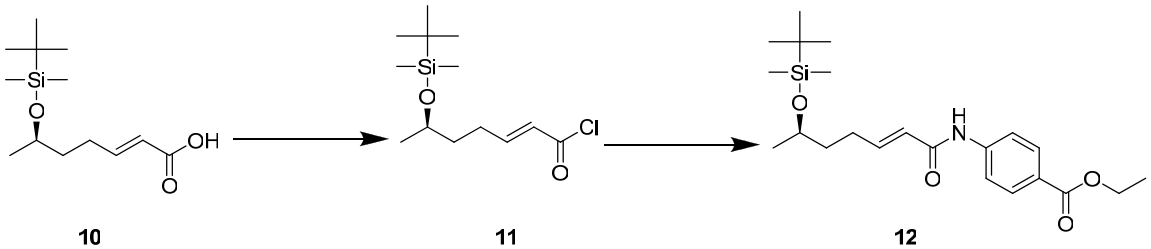
acetate:hexanes *{v/v}*), yielding **4** as a colorless oil (0.16 g, 0.93 mmol, 74%). ^{1}H NMR (600 MHz, acetone-d_6) δ 1.14 (d, *J* = 6.1 Hz, 3 H, 7-H), 1.23 (t, *J* = 7.2 Hz, 3 H, $CH_2C\underline{H}_3$), 1.52-1.56 (m, 2 H, 5-H), 2.25-2.31 (m, 1 H, 4-H), 2.34-2.39 (m, 1 H, 4-H), 3.71-3.77 (m, 1 H, 6-H), 4.12 (q, *J* = 7.1 Hz, 2 H, $C\underline{H}_2CH_3$), 5.83 (dt, *J* = 15.6, *J* = 1.6 Hz, 1 H, 2-H), 6.96 (dt, *J* = 15.6, *J* = 7.0 Hz, 1 H, 3-H).

6.6. 6*R*-(*t*-butyldimethylsiloxy)-2*E*-heptenoic acid



To a solution of **9** (0.43 g, 1.5 mmol) dissolved in dioxane (20 ml), aqueous lithium hydroxide (0.25 g, 6 mmol in 4 ml H_2O) was added and the resulting mixture was stirred at 60 °C 3 h. The resulting solution was concentrated *in vacuo* and re-suspended in ether (10 ml). The reaction was neutralized by adding a suspension of potassium dihydrogenphosphate (2.5 g) in water (10 ml), and the resulting biphasic mixture was extracted with two aliquots of ether (50 ml). Organic phases were combined and dried over sodium sulfate before being concentrated under reduced pressure, yielding 0.36 g (1.4 mmol, 93% yield) of **10** as a colorless oil.

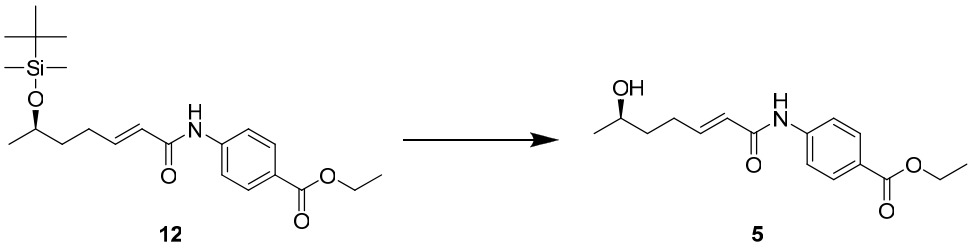
6.7. *N*-(6'*R*-[*t*-butyldimethylsiloxy]-2'*E*-heptenoyl)-4-aminobenzoic acid ethyl ester



Oxalylchloride (0.08g, 0.62 mmol) was added to a solution of **10** (0.09g, 0.35 mmol) in DCM (2 ml) at 0 °C. One drop of DMF (0.04 ml) was added, and the solution was stirred for 20 min at 0 °C. The solution was then concentrated *in vacuo* and redissolved in dry DCM (2 ml). After cooling to 0 °C, *p*-aminobenzoic acid (0.18 g, 1.1 mmol) followed by DIEA (0.18 g, 1.4 mmol) were added. The solution was stirred at 0 °C for 15 min, concentrated under reduced pressure, and purified by silica gel column chromatography (10:1 hexanes: ethyl acetate to 1:1 hexanes:

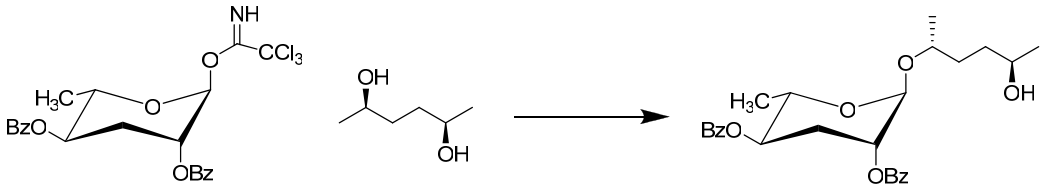
ethyl acetate *{v/v}*), yielding **12** (0.075 g, 0.19 mmol, 54%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.05 (s, 3 H, Si-CH_3), 0.06 (s, 3 H, Si-CH_3), 0.89 (s, 9 H, Si-C(CH_3)$_3$), 1.15 (d, *J* = 6.1 Hz, 3 H, 7-H), 1.38 (t, *J* = 7.1 Hz, 3 H, $CH_2C\underline{H}_3$), 1.53-1.65 (m, 2 H, 5-H), 2.20-2.40 (m, 2 H, 4-H), 3.80-3.88 (m, 1 H, 6-H), 4.36 (q, *J* = 7.1 Hz, 2 H, $C\underline{H}_2CH_3$), 5.93 (dt, *J* = 15.2, *J* = 1.6 Hz, 1 H, 2-H), 7.04 (dt, *J* = 15.2, *J* = 7.0 Hz, 1 H, 3-H), 7.25 (br. s, 1 H N-H), 7.63-7.66 (m, 2 H, *p*-aminobenzoyl), 8.00-8.03 (m, 2 H, *p*-aminobenzoyl).

6.8. *N*-(6'*R*-hydroxy-2'*E*-heptenoyl)-4-aminobenzoic acid ethyl ester



To a stirred solution of **12** (0.075 g, 0.19mmol) in acetonitrile (2 ml), one drop of 40% HF/H_2O was added at room temperature. Reaction progress was monitored using TLC (1:1 ethyl acetate: hexanes *{v/v}*) and was judged complete after 3 h. The solution was then neutralized with saturated aqueous sodium bicarbonate and extracted with three 3-ml-aliquots of DCM. Organic layers were combined and dried over sodium sulfate prior to concentration *in vacuo*. The residue was purified by silica column chromatography (1:5 to 1:1 ethyl acetate: hexanes *{v/v}*), yielding **5** (0.060 g, 0.19 mmol, 98%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.22 (d, *J* = 6.1 Hz, 3 H, 7-H), 1.38 (t, *J* = 7.1 Hz, 3 H, $CH_2C\underline{H}_3$), 1.59-1.65 (m, 2 H, 5-H), 2.23-2.42 (m, 2 H, 4-H), 3.84 (sext., *J* = 6.1 Hz, 1 H, 6-H), 4.35 (q, *J* = 7.1 Hz, 2 H, $C\underline{H}_2CH_3$), 5.98 (dt, *J* = 15.2, *J* = 1.6 Hz, 1 H, 2-H), 7.02 (dt, *J* = 15.2, *J* = 7.0 Hz, 1 H, 3-H), 7.60 (br. s, 1 H N-H), 7.65-7.67 (m, 2 H, *p*-aminobenzoyl), 7.99-8.02 (m, 2 H, *p*-aminobenzoyl).

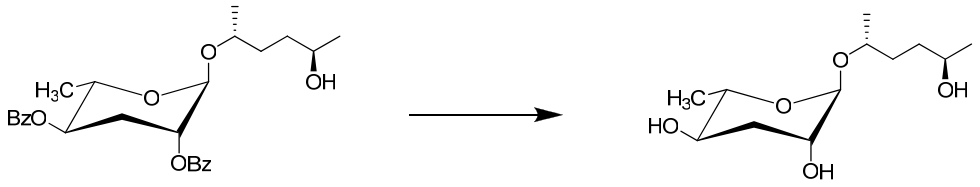
6.9. 5*R*-(3'*R*,5'*R*-dibenzoyloxy-6'*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy)-2*R*-hexanol



6 **13** **14**

A solution of **6** (1.02 g, 2 mmol)[6] and 2*R*,5*R*-hexanediol (**13**, 0.59 g, 5.0 mmol) in dry DCM (5 ml) under argon was cooled to 0 °C. Trimethylsilyl trifluoromethylsulfonate ([TMSOTF] 0.045 g, 0.20 mmol) was added via syringe at 0 °C. Reaction progress was monitored by TLC (1:2 ethyl acetate: hexanes *{v/v}*) and judged complete after 2 h. The solution was allowed to warm to room temperature and was neutralized with saturated aqueous sodium bicarbonate solution (2 ml). The mixture was extracted with 1-ml-three portions of DCM and the organic phases were combined and dried over sodium sulfate prior to purification by silica gel column chromatography (1:5 ethyl acetate:hexanes to 1:1 ethyl acetate: hexanes *{v/v}*), yielding **14** (0.66 g, 1.43 mmol, 72%) as a colorless oil. ^{1}H NMR (600 MHz, benzene-d_6) δ 1.00 (d, J = 6.1 Hz, 3 H, 6-H), 1.015 (d, J = 6.1 Hz, 3 H, 1-H), 1.35 (d, J = 6.3 Hz, 3 H, 6'-CH_3), 1.33-1.48 (m, 3 H), 1.64-1.72 (m, 1 H), 2.25 (ddd, J = 13.8, J = 11.5, J = 3.1 Hz, 1 H, 4'-H_{ax}), 2.35-2.56 (m, 1 H, 4'-H_{eq}), 3.55-4.60 (m, 1 H, 2-H), 3.75-3.80 (m, 1 H, 5-H), 4.35 (dq, J = 9.8, J = 6.3 Hz, 1 H, 6'-H), 4.31 (q, J = 7.1 Hz, 2 H, C$\underline{H}_2$CH$_3$), 5.17 (br. s, 1 H 2'-H), 5.35 (td, J = 3.1, J = 1.5 Hz, 1 H, 3'-H), 5.64, (ddd, J = 11.3, J = 9.8, J = 4.8 Hz, 1 H 5'-H), 7.02-7.07 (m, 4 H, benzoyl), 7.10-7.15 (m, 2 H, benzoyl), 8.11-8.13 (m, 2 H, benzoyl), 8.31-8.33 (m, 2 H, benzoyl).

6.10. 5*R*-(3'*R*,5'*R*-dihydroxy-6'*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy)-2*R*-hexanol (ascr#6.1)

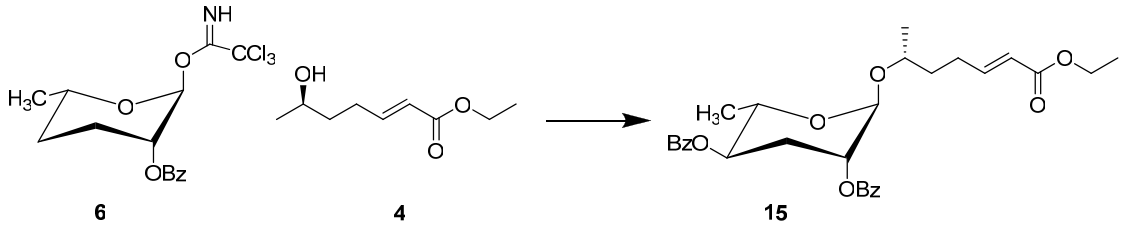


14 **ascr#6.1**

A solution of **14** (0.33 g, 0.72 mmol) was dissolved in methanol (4 ml) and 4 ml of a 0.5 M aqueous potassium hydroxide solution was added . Reaction progress was monitored by TLC (8:1 dichloromethane: methanol *{v/v}*). Reaction was judged complete after 2 h, at which point

sodium bicarbonate (5 mg) was added to buffer the basic solution. The reaction mixture was filtered over a cotton plug and the filtrate concentrated *in vacuo* followed by silica gel column chromatography (12:1 DCM:MeOH to 2:1 DCM: MeOH *{v/v}*), yielding **ascr#6.1** (0.16 g, 0.66 mmol, 93%) as a colorless oil, $[\alpha]_D^{20}$ = -101, *c* 0.52 (methanol). For NMR spectroscopic data see Supporting Table S1.

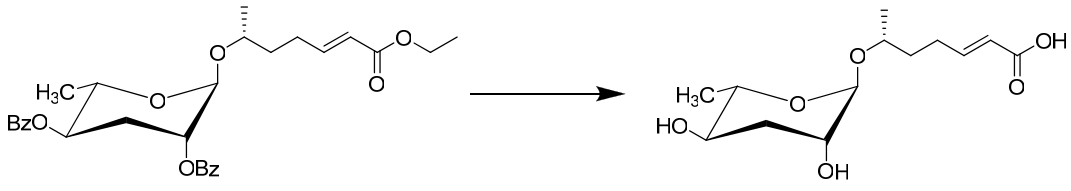
6.11. 6*R*-(3'*R*,5'*R*-dibenzoyloxy-6'*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy)-2*E*-heptenoic acid ethyl ester



6 **4** **15**

To a stirred solution of **6** (0.33 g, 0.66 mmol)[6] and **4** (0.13 g, 0.75 mmol) in dry DCM (5 ml) at 0 °C under argon TMSOTF (0.02 g, 0.083 mmol) was added *via* syringe. Reaction progress was monitored by TLC (1:1 ethyl acetate: hexanes *{v/v}*) and judged complete after 4 h. The reaction mixture was allowed to warm to room temperature gradually and was neutralized with saturated aqueous sodium bicarbonate solution (2 ml). The mixture was extracted with three portions of DCM (1 ml), and the organic phases were combined and dried over sodium sulfate prior to purification by silica gel column chromatography (1:4 ethyl acetate:hexanes to 2:1 ethyl acetate: hexanes *{v/v}*), yielding **15** (0.33 g, 0.24 mmol, 36%) as a colorless oil. In addition, 60 mg (45%) of unreacted **4** was recovered.

6.12. 6*R*-(3'*R*,5'*R*-dihydroxy-6'*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy)-2*E*-heptenoic acid

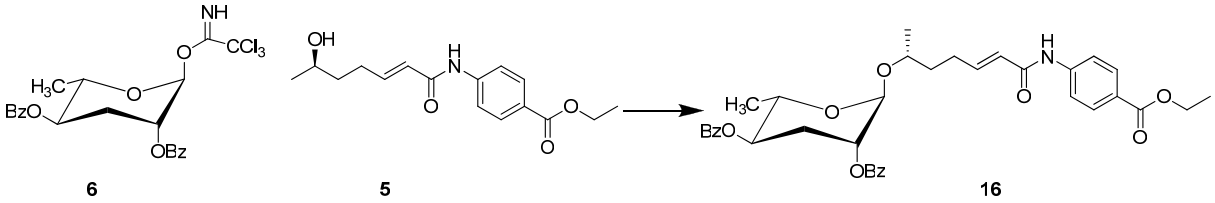


15 **ascr#7**

To a solution of **15** (0.33 g, 0.24 mmol) dissolved in dioxane (10 ml) and dry THF (6 ml), aqueous lithium hydroxide (10 ml 0.5 M solution) was added, and the resulting mixture was stirred at 70 °C for 3 h. After cooling to room temperature, glacial acetic acid (0.60 g, 10 mmol)

was added with stirring. The solution was concentrated under reduced pressure and purified by silica gel column chromatography (19:1 DCM:methanol to 6:1 DCM:methanol *{v/v}*), yielding **ascr#7** (0.065 g, 0.24 mmol, 100%) as a colorless oil, $[\alpha]_D^{20}$ = -85.3, *c* 3.2 (methanol). For NMR spectroscopic data see Supporting Table S3.

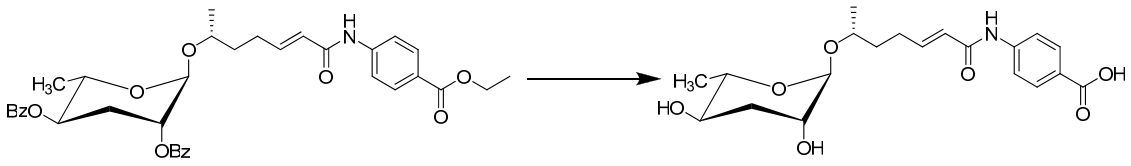
6.13. *N*-(6'*R*-[3''*R*,5''*R*-dibenzoyloxy-6''*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy]-2'*E*-heptenoyl)-4-aminobenzoic acid ethyl ester



6 **5** **16**

To a stirred solution of **6** (0.13g, 0.25 mmol) and **5** (0.067g, 0.23 mmol) in dry DCM (2 ml) at 0 °C, TMSOTF (0.005 g, 0.025 mmol) was added. Reaction progress was monitored by TLC (1:1 ethyl acetate: hexanes *{v/v}*) and judged complete after 2 h. The reaction mixture was allowed to warm to room temperature and was neutralized with saturated aqueous sodium bicarbonate solution (1 ml). The mixture was extracted with 1-ml-three portions of DCM, and the organic phases were combined and dried over sodium sulfate prior to purification by silica gel column chromatography (1:10 ethyl acetate:hexanes to 1:1 ethyl acetate:hexanes *{v/v}*), yielding **16** (140 mg, 0.22 mmol, 89 %) as a white powder. ^{1}H NMR (500 MHz, acetone-d_6) δ 1.25 (d, *J* = 6.1 Hz, 3 H, 7-H), 1.27 (d, *J* = 6.3 Hz, 3 H, 6'-CH_3), 1.35 (t, *J* = 7.2 Hz, 3 H, $CH_2C\underline{H}_3$), 1.73-1.88 (m, 2 H, 5-H), 2.26 (ddd, *J* = 14, *J* = 11.5, *J* = 3.0 Hz, 1 H, 4'-H_{ax}), 2.35-2.56 (m, 3 H, 4-H, 4'-H_{eq}), 3.95-4.02 (m, 1 H, 6-H), 4.20 (dq, *J* = 9.8, *J* = 6.3 Hz, 1 H, 6'-H), 4.31 (q, *J* = 7.1 Hz, 2 H, $C\underline{H}_2CH_3$), 5.03 (br. s, 1 H 2'-H), 5.12-5.18 (m, 2 H, 3'-H and 5'-H), 6.23 (dt, *J* = 15.1, *J* = 1.6 Hz, 1 H, 2-H), 7.07 (dt, *J* = 15.1, *J* = 7.0 Hz, 1 H, 3-H), 7.49-7.55 (m, 2 H, benzoyl), 7.55-7.60 (m, 2 H, benzoyl), 7.63-7.67 (m, 1 H, benzoyl), 7.67-7.72 (m, 1 H, benzoyl), 7.83-7.85 (m, 2 H, *p*-aminobenzoyl), 7.94-7.96 (m, 2 H, *p*-aminobenzoyl), 8.03-8.05 (m, 2 H, benzoyl), 8.10-8.12 (m, 2 H, benzoyl).

6.14. *N*-(6'*R*-[3''*R*,5''*R*-dihydroxy-6''*S*-methyl-(*2H*)-tetrahydropyran-2-yloxy]-2'*E*-heptenoyl)-4-aminobenzoic acid (ascr#8)



To a stirred solution of **16** (20 mg, 0.03 mmol) in dioxane (1 ml), 1 M aqueous lithium hydroxide solution (0.4 ml) and water (0.6 ml) were added and the resulting mixture was stirred at 62 °C for 3 h. After cooling to room temperature, a solution of potassium dihydrogen phosphate (0.5 g) in water (10 ml) was added. The entire mixture was concentrated *in vacuo*, re-suspended in methanol and evaporated onto Celite®. Silica gel column chromatography (20:1 DCM: methanol to 3:1 DCM: methanol *{v/v}*) yielded **ascr#8** (12 mg, 0.03 mmol, ~100 %) as a white solid. ^{1}H NMR spectroscopic analysis indicated the presence of 4-8% of the *cis*-isomer. Therefore, the material was further purified *via* reversed-phase HPLC, using aqueous ammonium acetate and methanol as solvents, as described in the following section 7. Fractions containing **ascr#8** were pooled and re-chromatographed (silica gel column chromatography using 20:1 DCM:methanol to 3:1 DCM:methanol *{v/v}*)) in order to remove ammonium acetate. Yield: 8 mg (0.02 mmol), $[\alpha]_D^{20}$ = -48.0, *c* 0.35 (methanol). For NMR spectroscopic data see Table S4.

7. Mass spectrometric analysis of metabolite extracts

Metabolite extracts derived from 250 mL of *daf-22* and N2 worm conditioned media were re-suspended in 200 µL of 1:1 water:methanol mixture. The suspension was centrifuged, and 10 µl aliquots of the supernatant were submitted to HPLC-MS, using the Agilent HPLC system equipped with a Supelco Discovery HS C-18 column (25 cm x 10 mm, 5 µm particle diameter). A 40 mM ammonium acetate buffer-methanol solvent gradient was used, starting with a methanol content of 20%, which was increased to 100% over a period 30 min. For reference, synthetic samples of ascr#2, ascr#3, ascr#6.1, ascr#7 and ascr#8 were analyzed using the same method (Fig. S12-S16). These HPLC-MS analyses showed that wild-type liquid cultures contained variable amounts of ascarosides, generally in the range of 10-100 nM for ascr#1, 100-200 nM for ascr#2, 100-200 nM for ascr#3, 10-20 nM for ascr#6.1 and ascr#6.2, 10-40 nM for ascr#7, and 10-70 nM for ascr#8.

8. References

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Supporting Figures

A shortcut to identifying small molecule signals that regulate behavior and development in *Caenorhabditis elegans*

Chirag Pungaliya[1], Jagan Srinivasan[2], Bennett W. Fox[1], Rabia U. Malik[1], Andreas H. Ludewig[1], Paul W. Sternberg[2] and Frank C. Schroeder[1]*

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Supporting Figures

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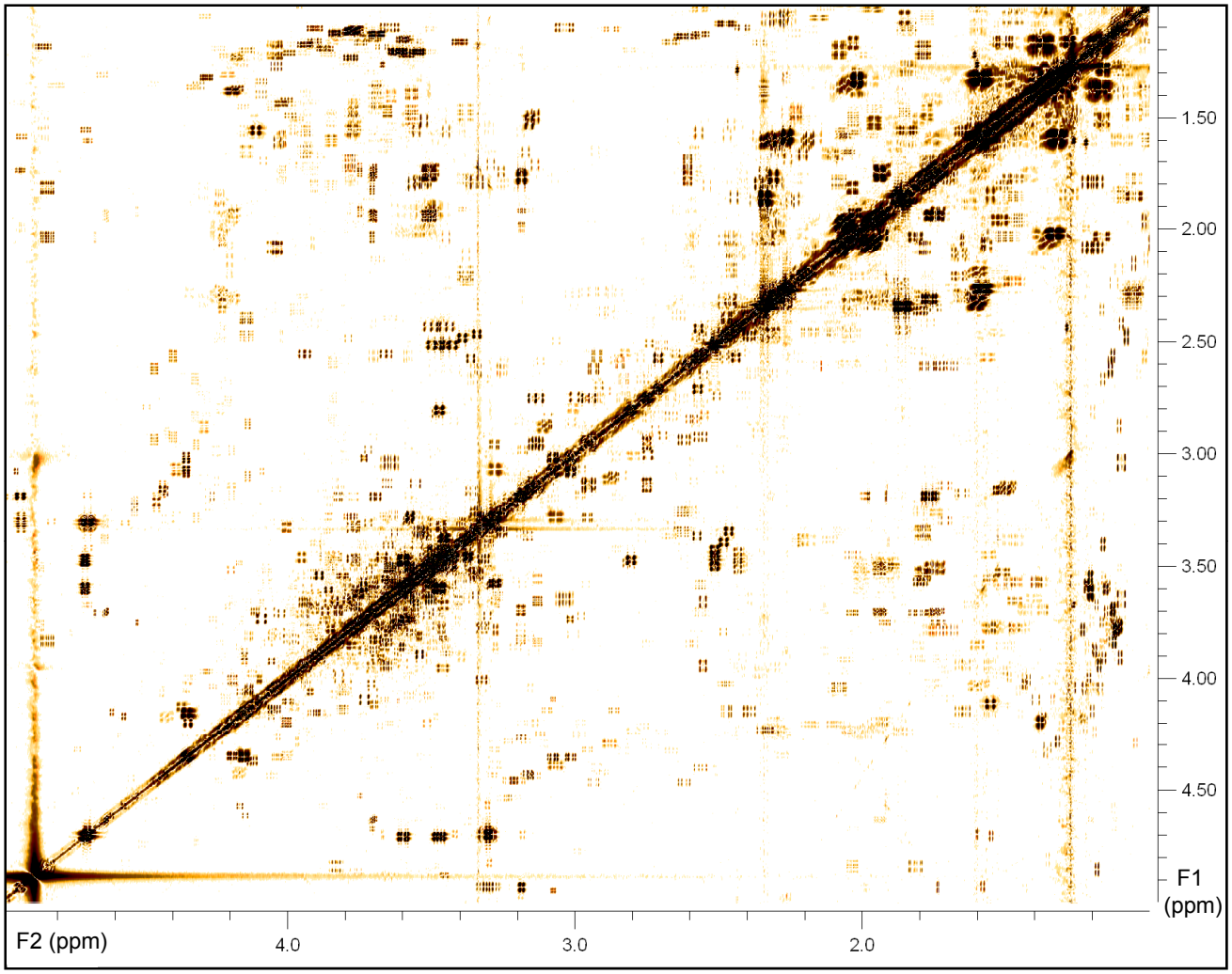
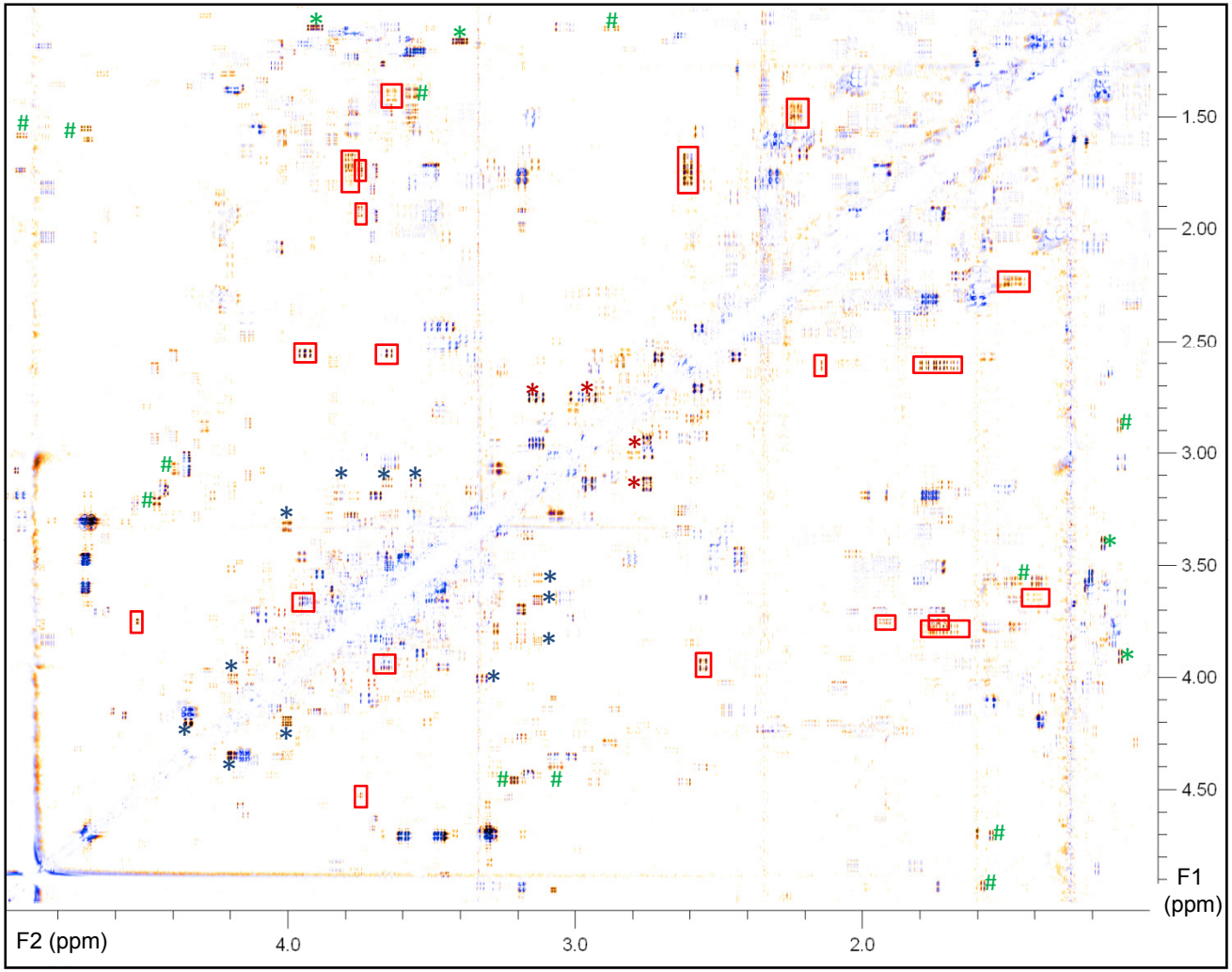
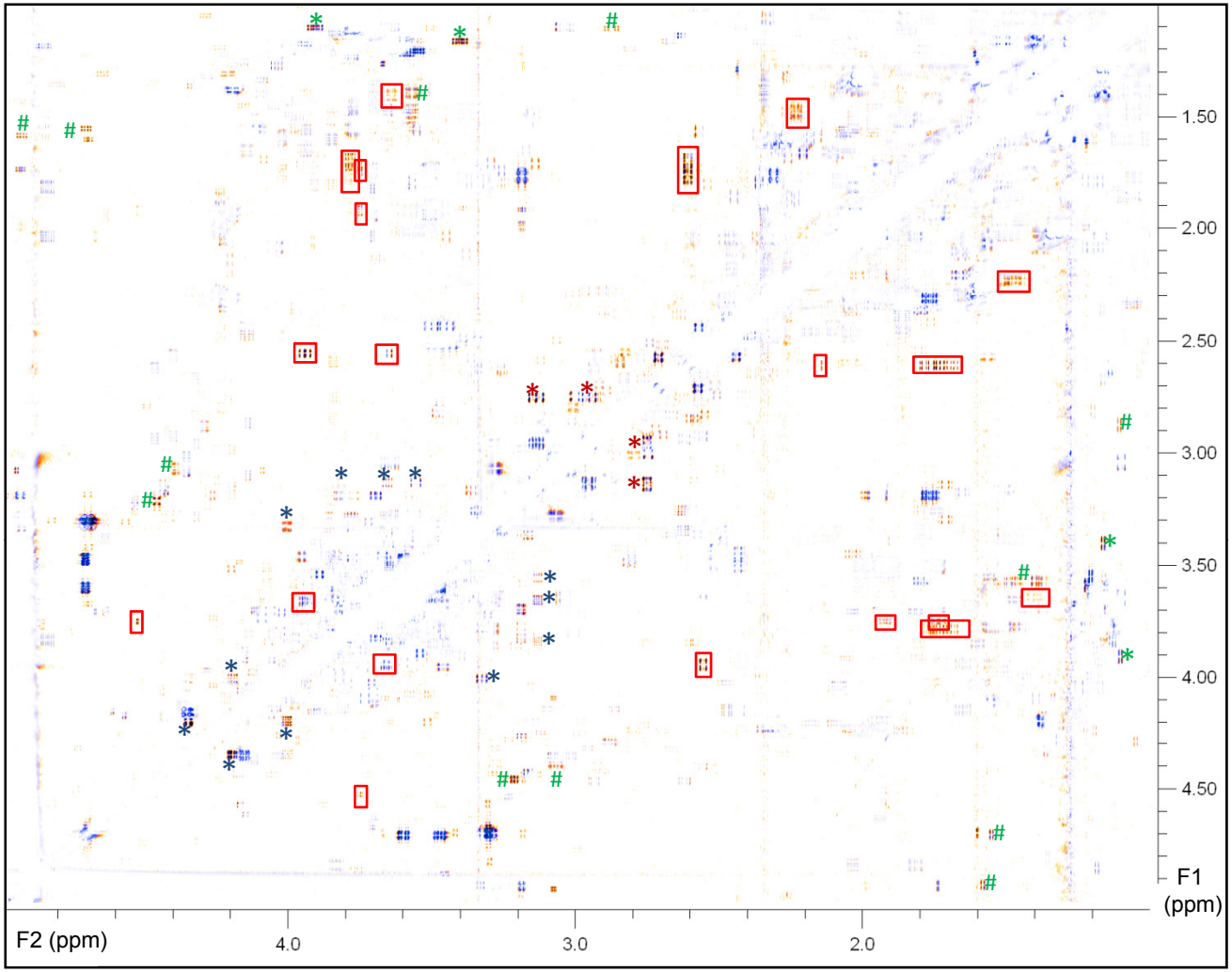


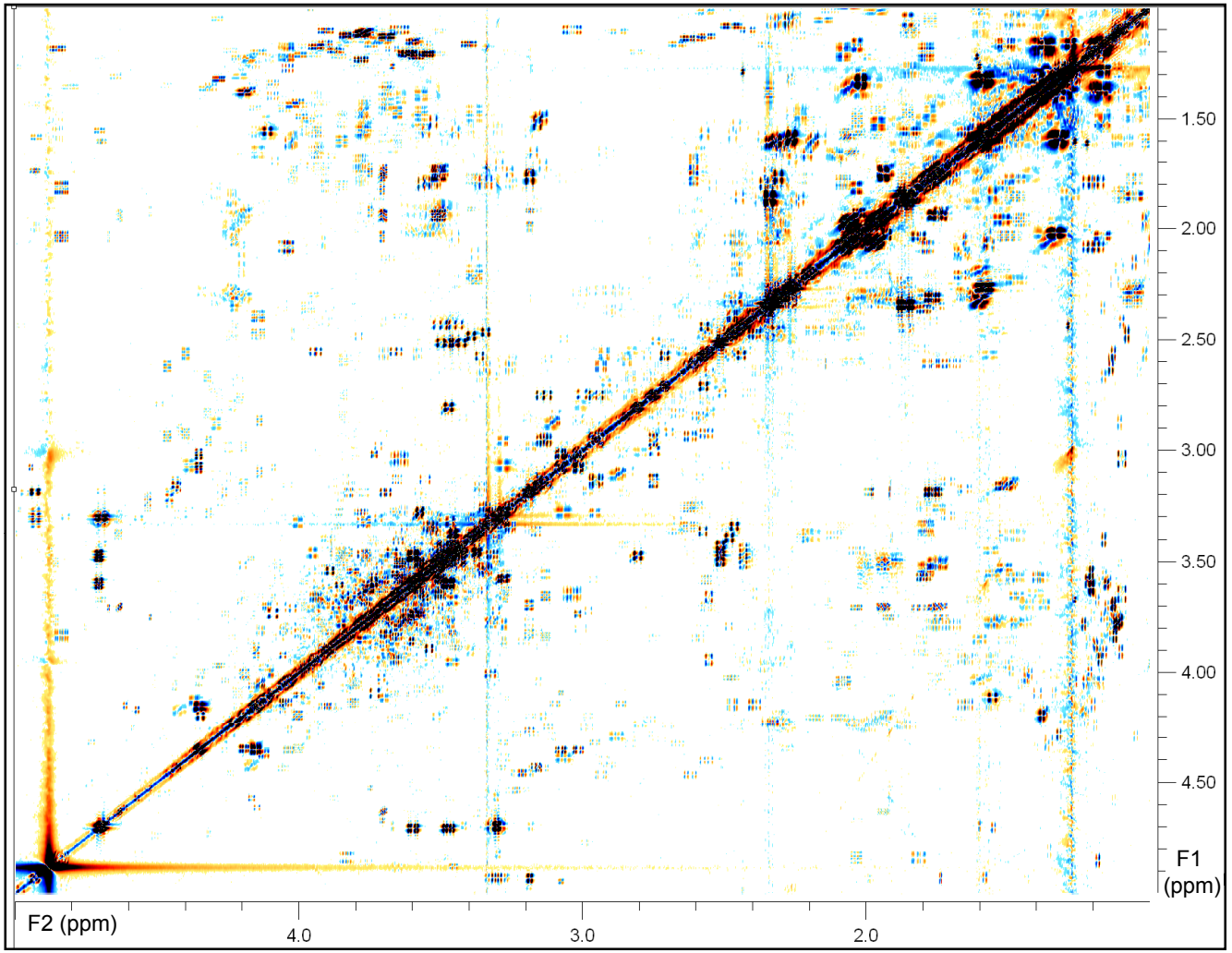
Supporting Figure 1. Absolute-value representation of the 1-5 ppm section from the dqfCOSY spectrum of wild-type (N2) metabolite extract.



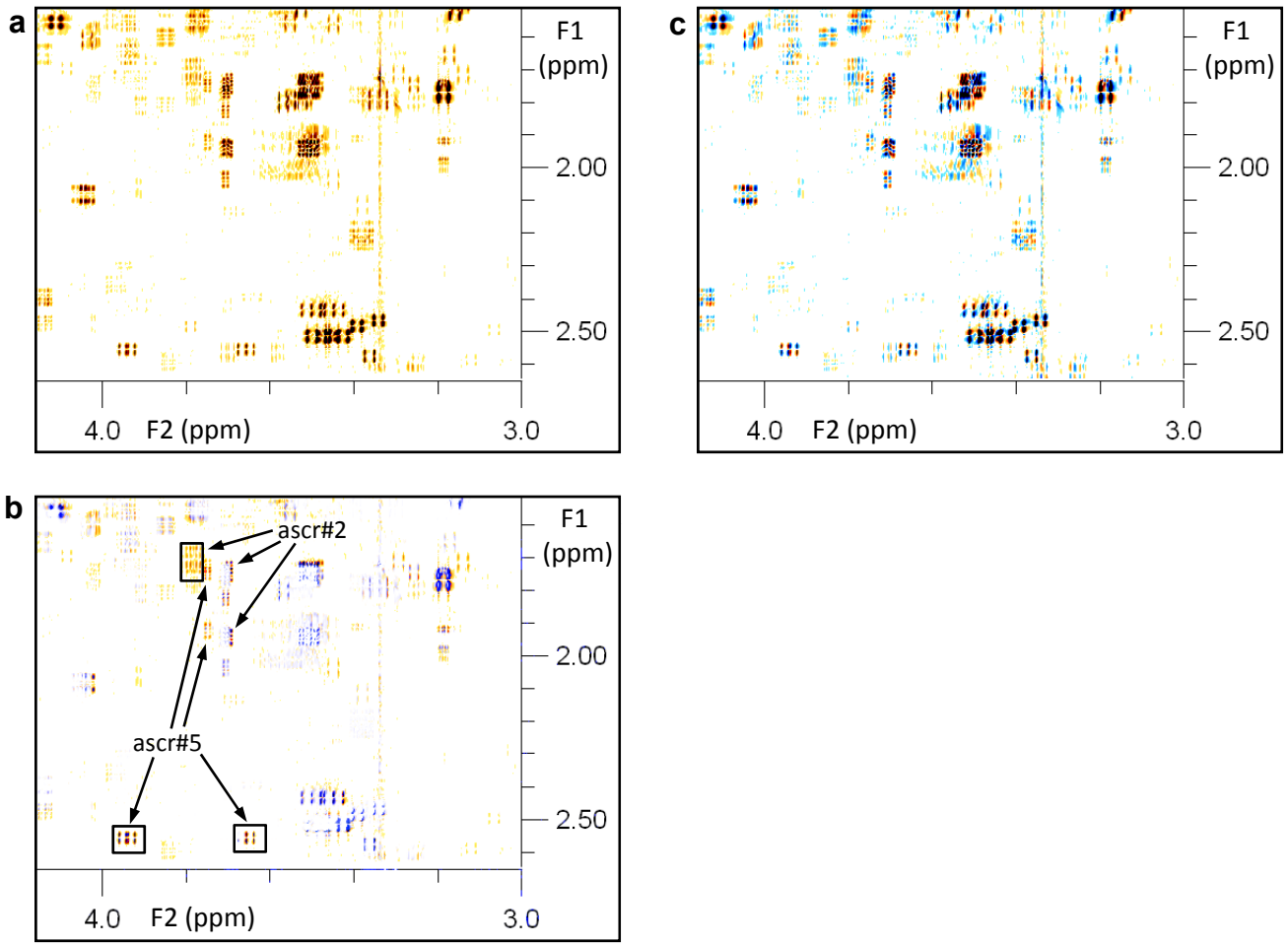
Supporting Figure 2a. DANS overlay of absolute-value dqfCOSY spectra from wild-type and *daf-22* metabolite extracts, revealing compounds present in wildtype but absent in *daf-22*. Signals present in both wildtype and *daf-22* spectra cancel or change color (blue), whereas signals representing compounds only present in wildtype remain unaffected (brown). Signals representing ascarosides are marked red. Several other groups of signals represent compounds not consistently expressed in either wildtype or *daf-22*, including an α-*D*-glucose derivative (marked with a blue asterisk "*"), a β-alanine derivative (marked with a red asterisk "*"), alpha- and β-*L*-rhamnose (marked with a green asterisk "*"), as well as several other amino acid derivatives (marked with a green pound sign "#"). Several other crosspeaks representing compounds present in both wild-type and *daf-22* extracts appear incompletely suppressed because of small sample-specific differences in chemical shift values and as a result of the reduced resolution of the display.



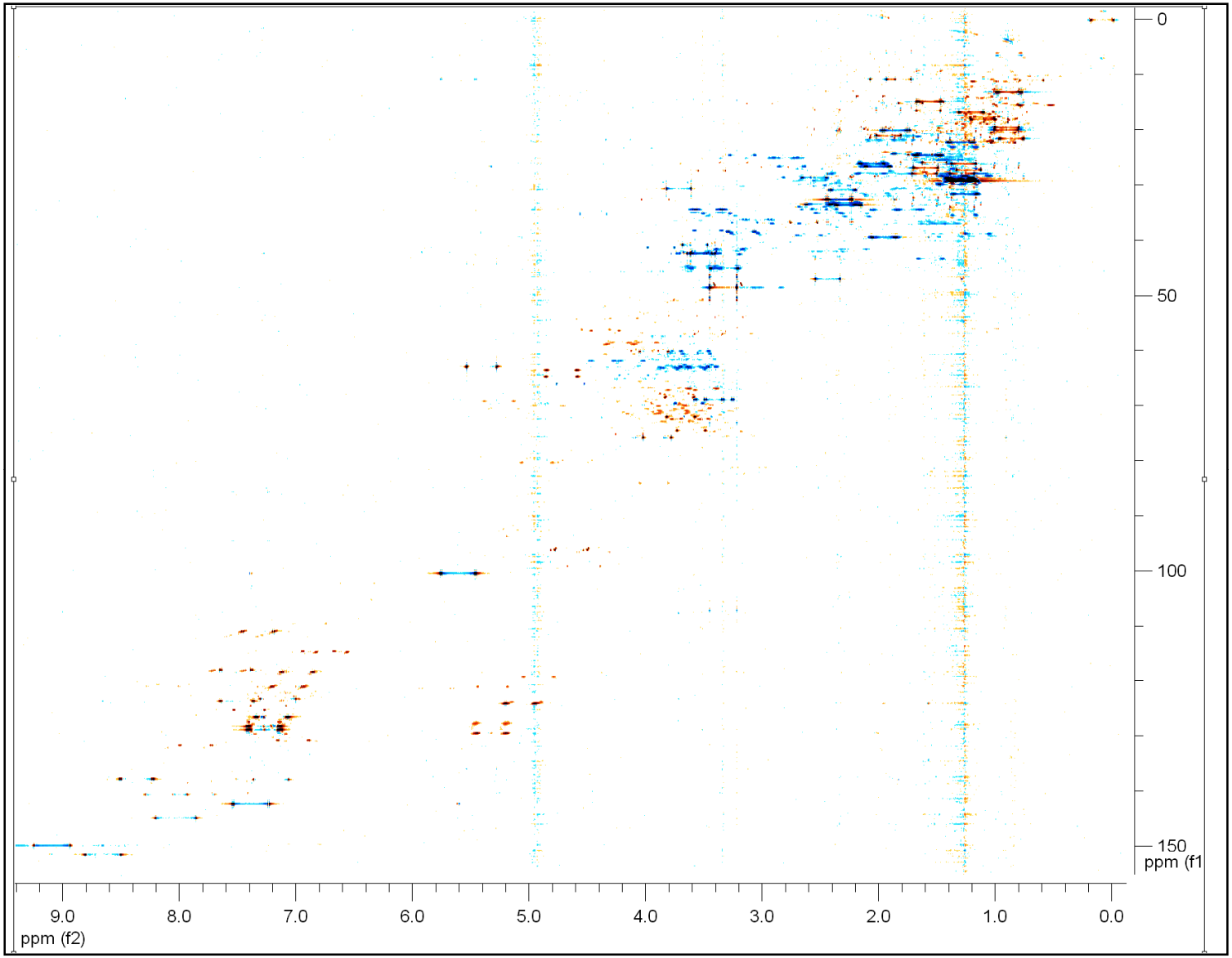
Supporting Figure 2b. Enhanced DANS overlay of absolute-value dqfCOSY spectra from wild-type and *daf-22* metabolite extracts. A second *daf-22* spectrum was superimposed on the spectrum shown in Supporting Figure 2a, resulting in better signal suppression. The second *daf-22* spectrum was shifted by 5 Hz up-field in both dimensions, in order to compensate for concentration-dependent chemical shift changes. Especially amino acid derivatives are suppressed better.



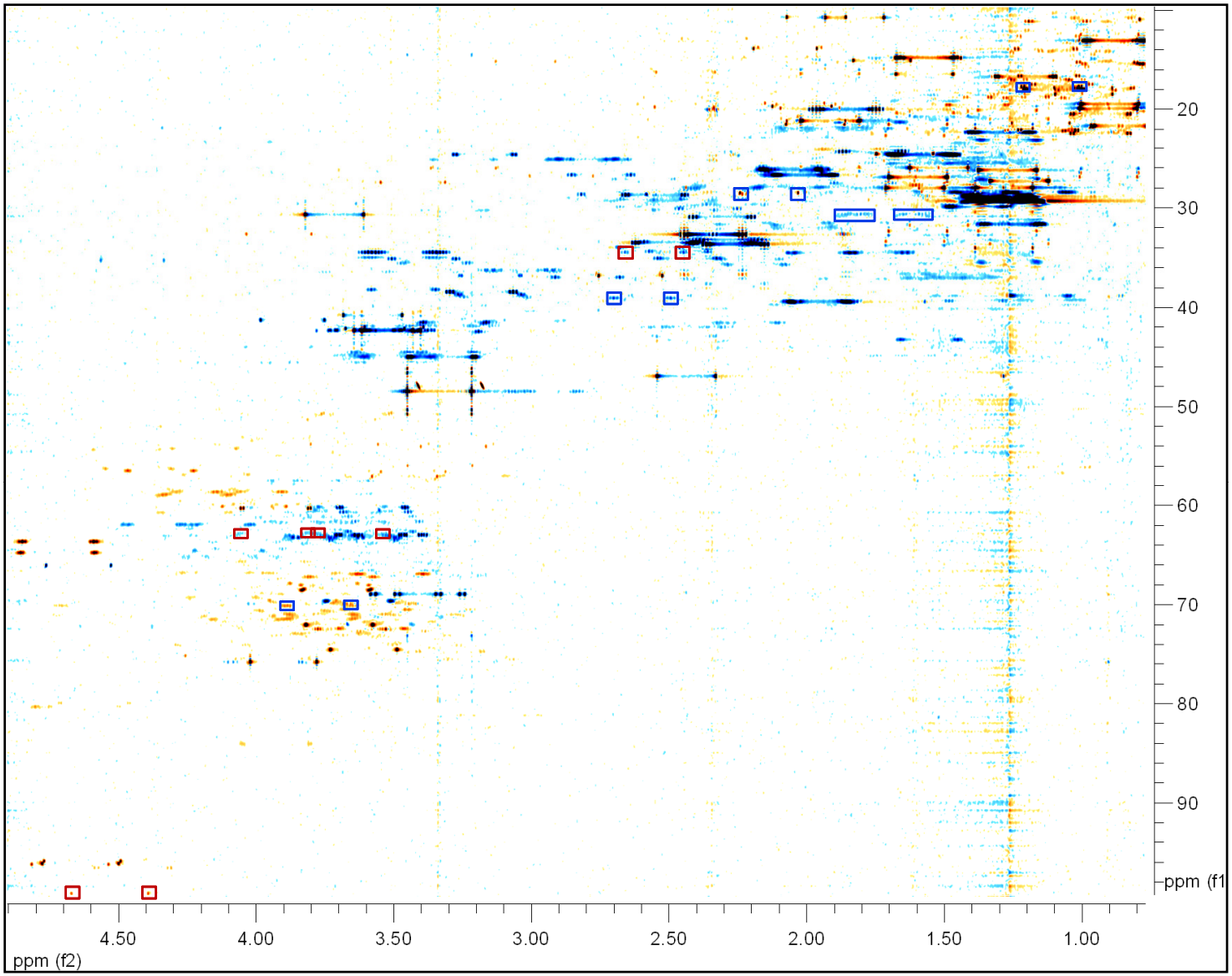
Supporting Figure 3. Signals detected via DANS were characterized further using the phase-sensitive representation of the dqfCOSY spectrum of wild-type (N2) metabolite extract.



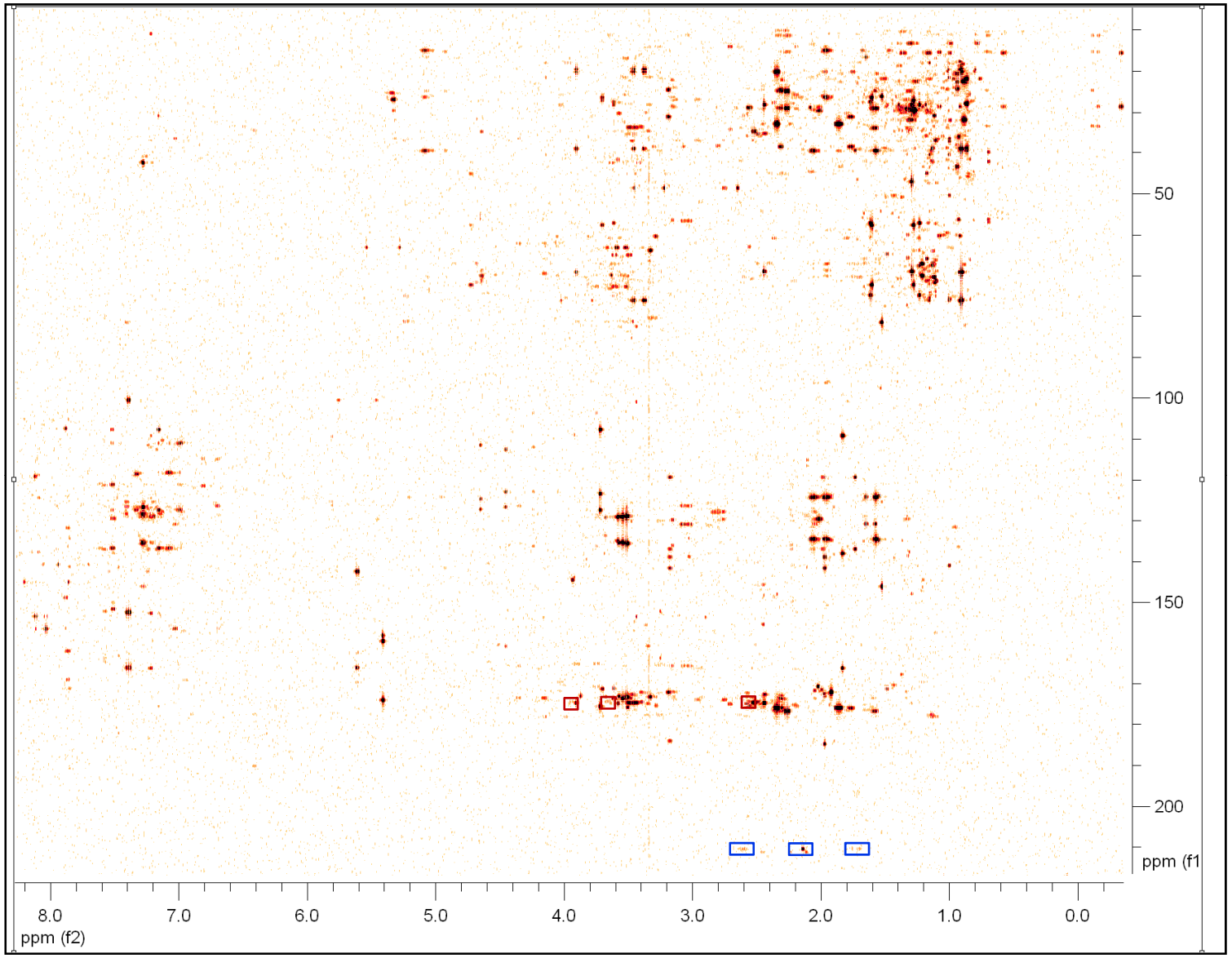
Supporting Figure 4. a: Section of absolute-value mode display of the dqfCOSY spectrum obtained form wild-type metabolite extract. **b**: Partial display of DANS overlay of absolute-value mode dqfCOSY spectra from wild-type and daf-22 metabolite extracts. Signals present in both wildtype and *daf-22* spectra cancel or change color (blue), whereas signals representing compounds only present in wildtype remain unaffected (brown). Signals representing ascarosides are marked. **c**: Signals detected *via* DANS were characterized further using the phase-sensitive representation of the dqfCOSY spectrum in **a**.



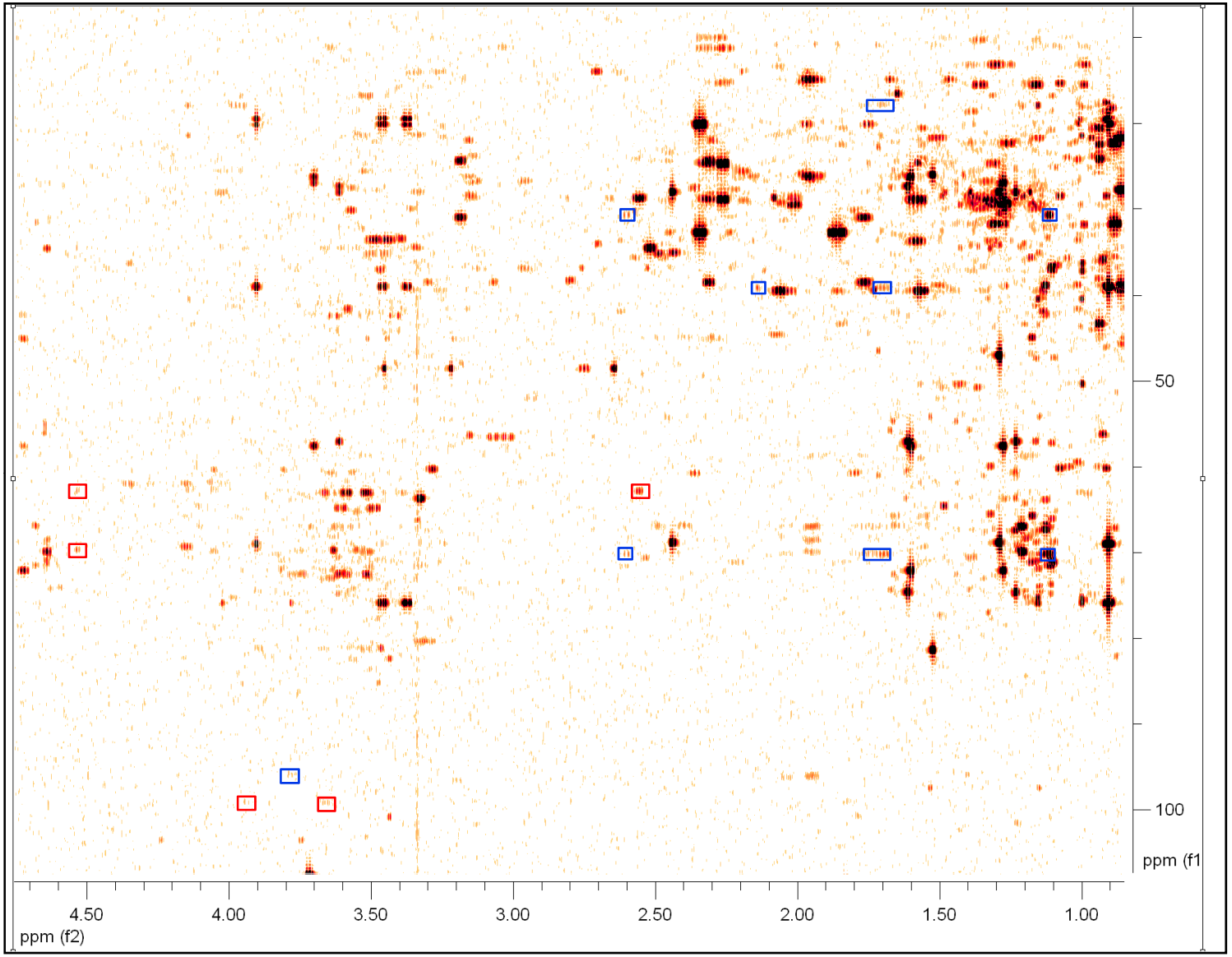
Supporting Figure 5. Phase-sensitive HSQC spectrum of wild-type (N2) metabolite extract.



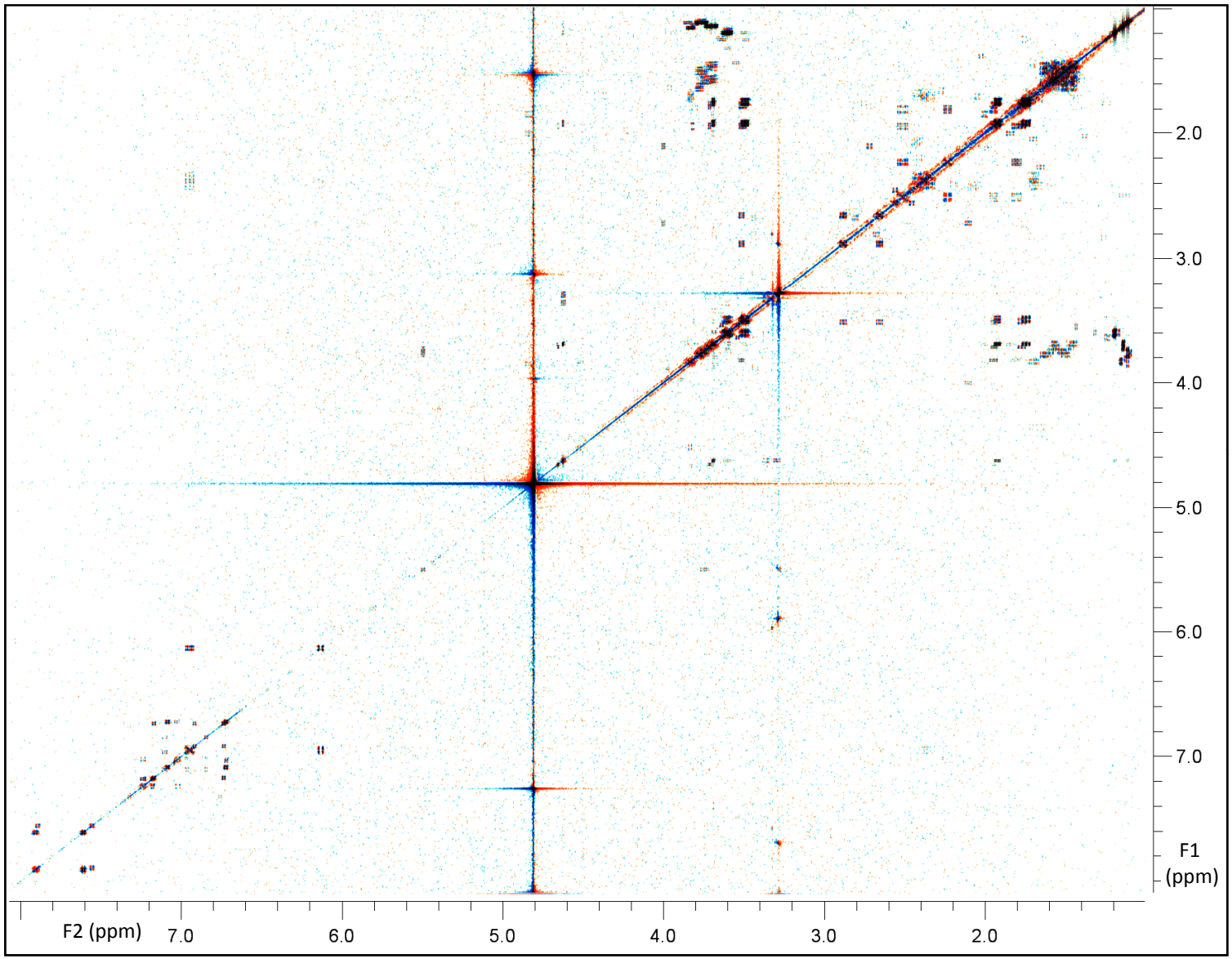
Supporting Figure 6. 0.8-4.9 ppm section of the phase-sensitive HSQC spectrum of wild-type (N2) metabolite extract in Supporting Figure 5. Marked signals represent the side chains of ascr#2 (blue) and ascr#5 (red).



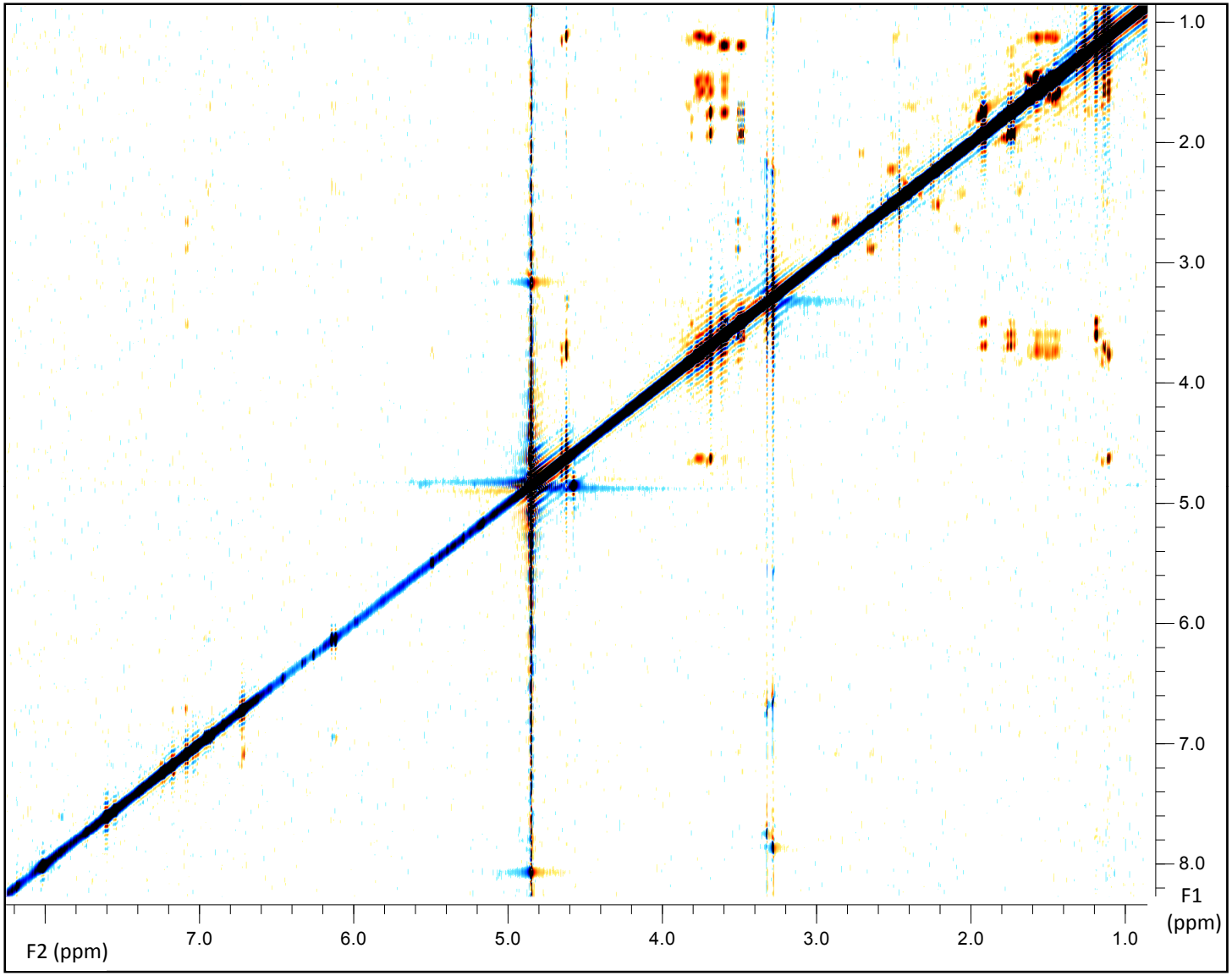
Supporting Figure 7. Magnitude-mode HMBC spectrum of wild-type (N2) metabolite extract. Marked signals represent the side chains of ascr#2 (blue) and ascr#5 (red).



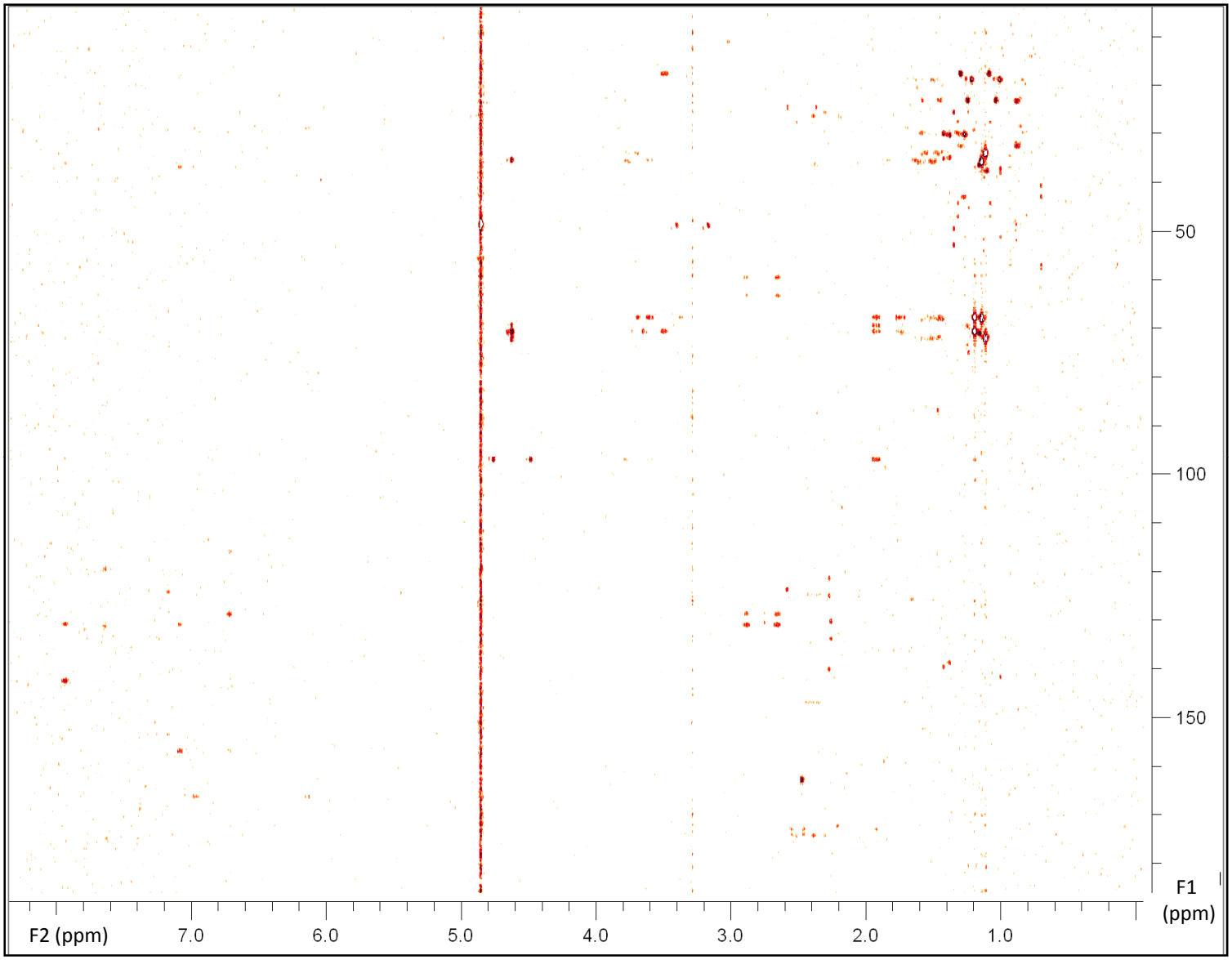
Supporting Figure 8. 0.85-4.75 ppm section of the magnitude-mode HMBC spectrum of wild-type (N2) metabolite extract in Supporting Figure 7. Marked signals represent the side chains of ascr#2 (blue) and ascr#5 (red).



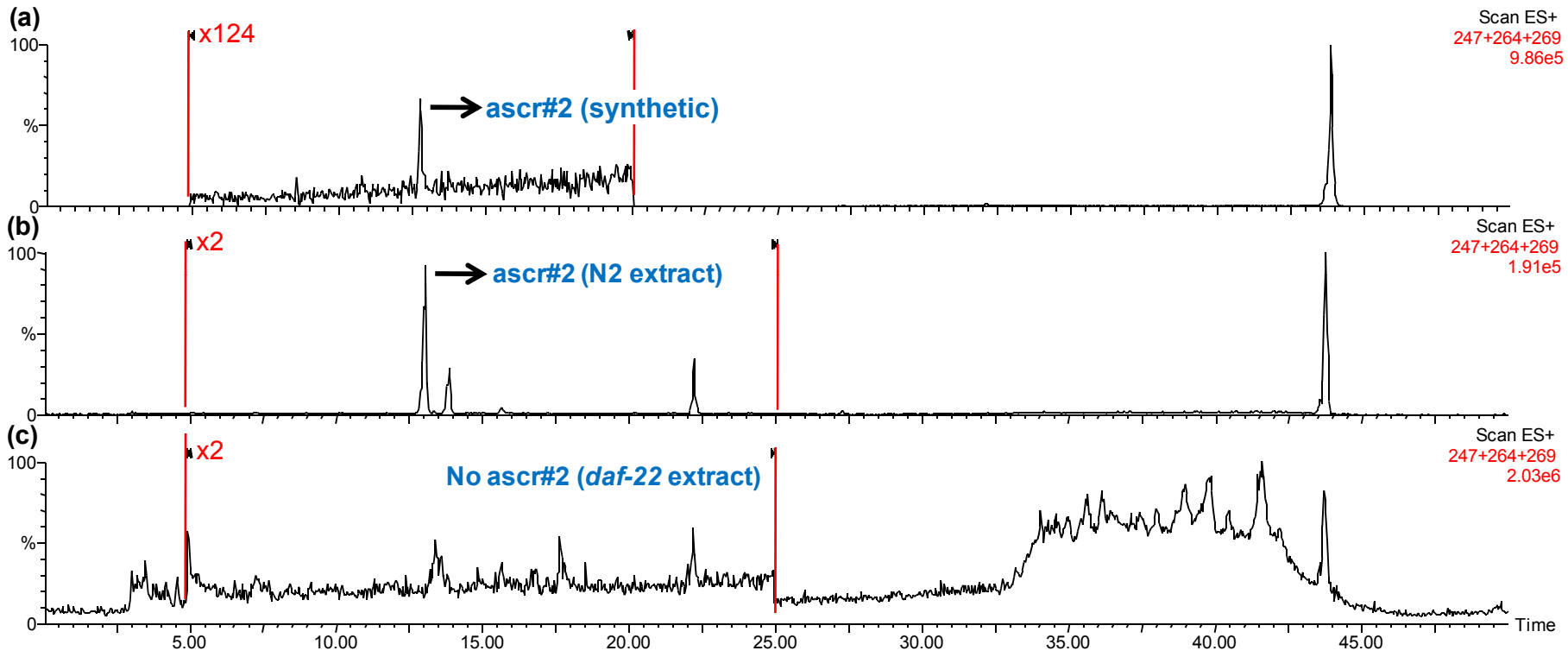
Supporting Figure 9. dqfCOSY spectrum of wild-type metabolite fraction containing ascr#6.1, ascr#6.2, and ascr#8 as major components.



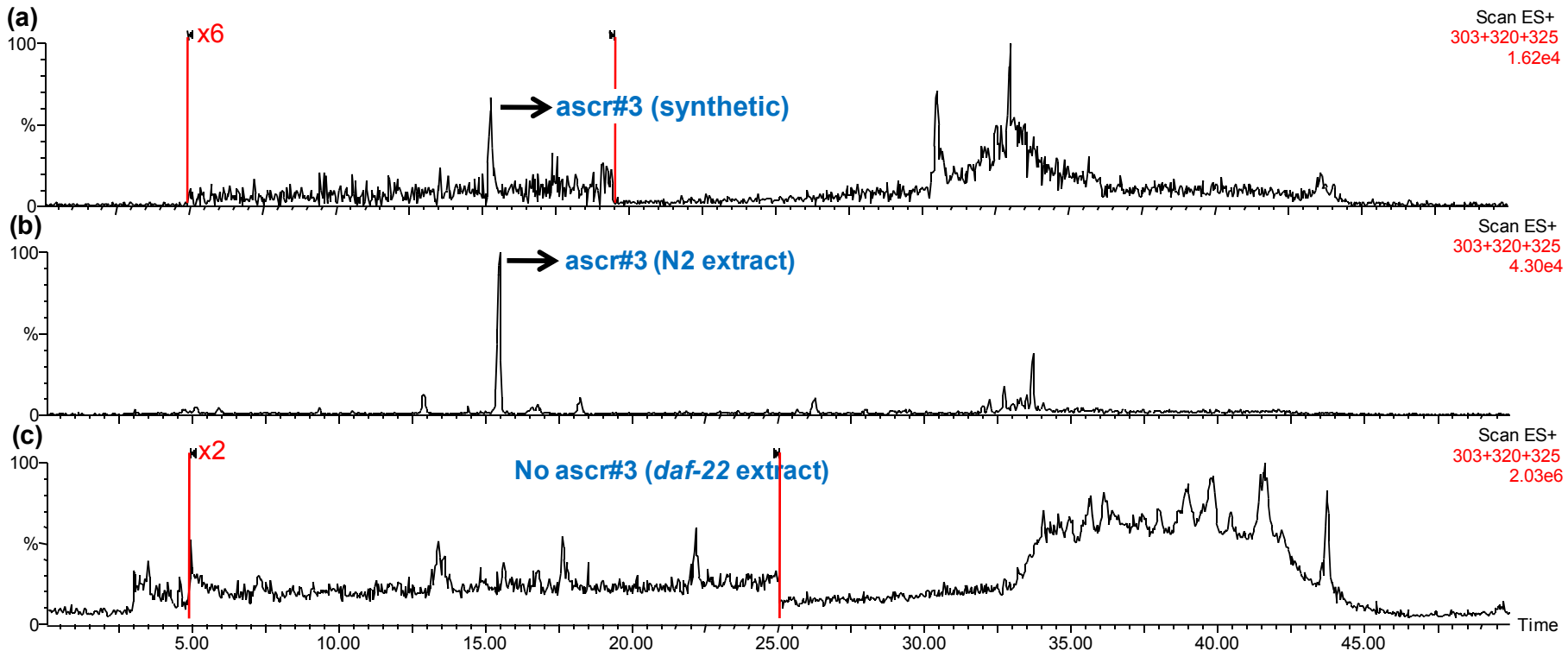
Supporting Figure 10. NOESY spectrum of wild-type metabolite fraction containing ascr#6.1, ascr#6.2, and ascr#8 as major components.



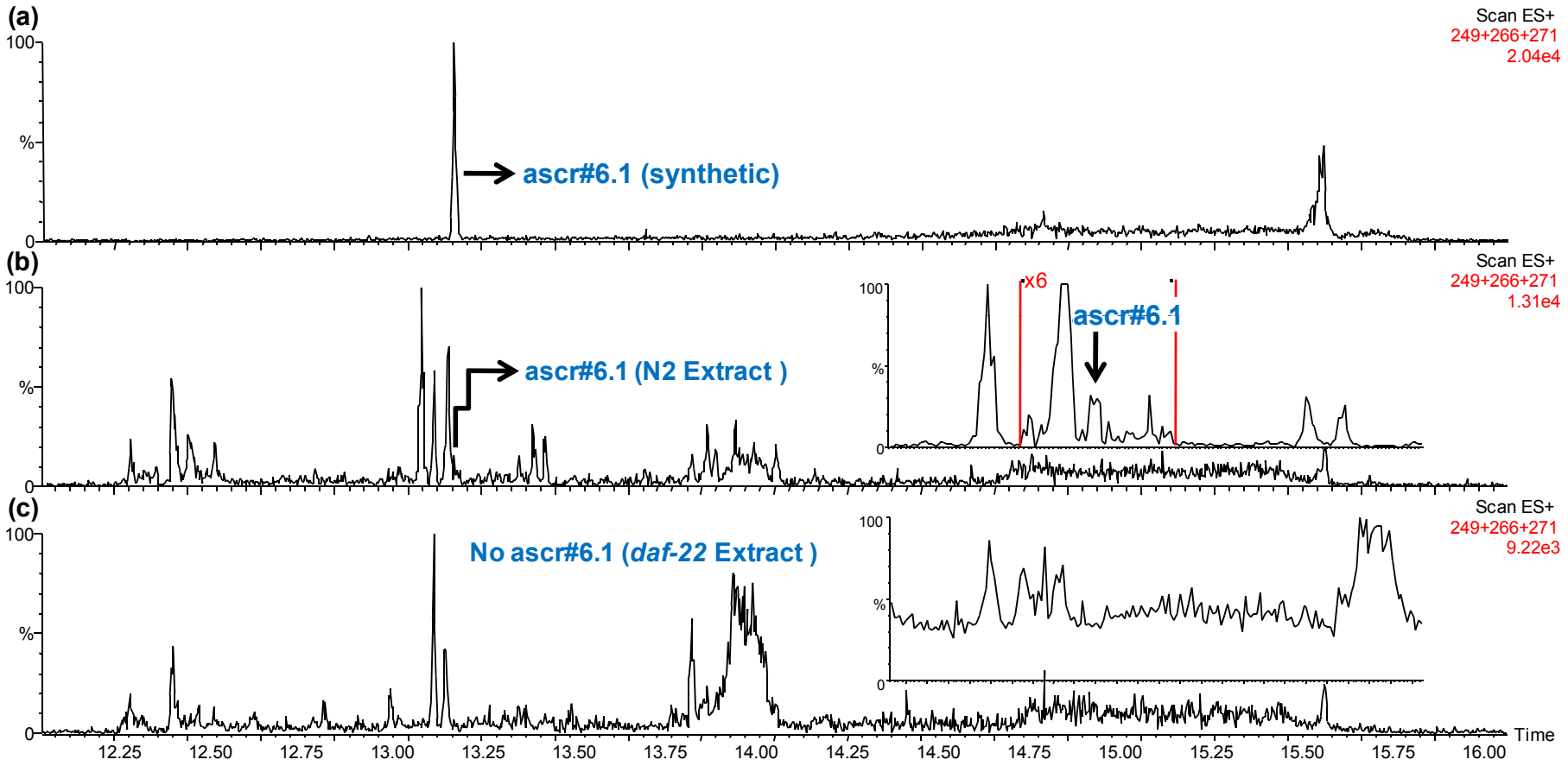
Supporting Figure 11. HMBC spectrum of wild-type metabolite fraction containing ascr#6.1, ascr#6.2, and ascr#8 as major components.



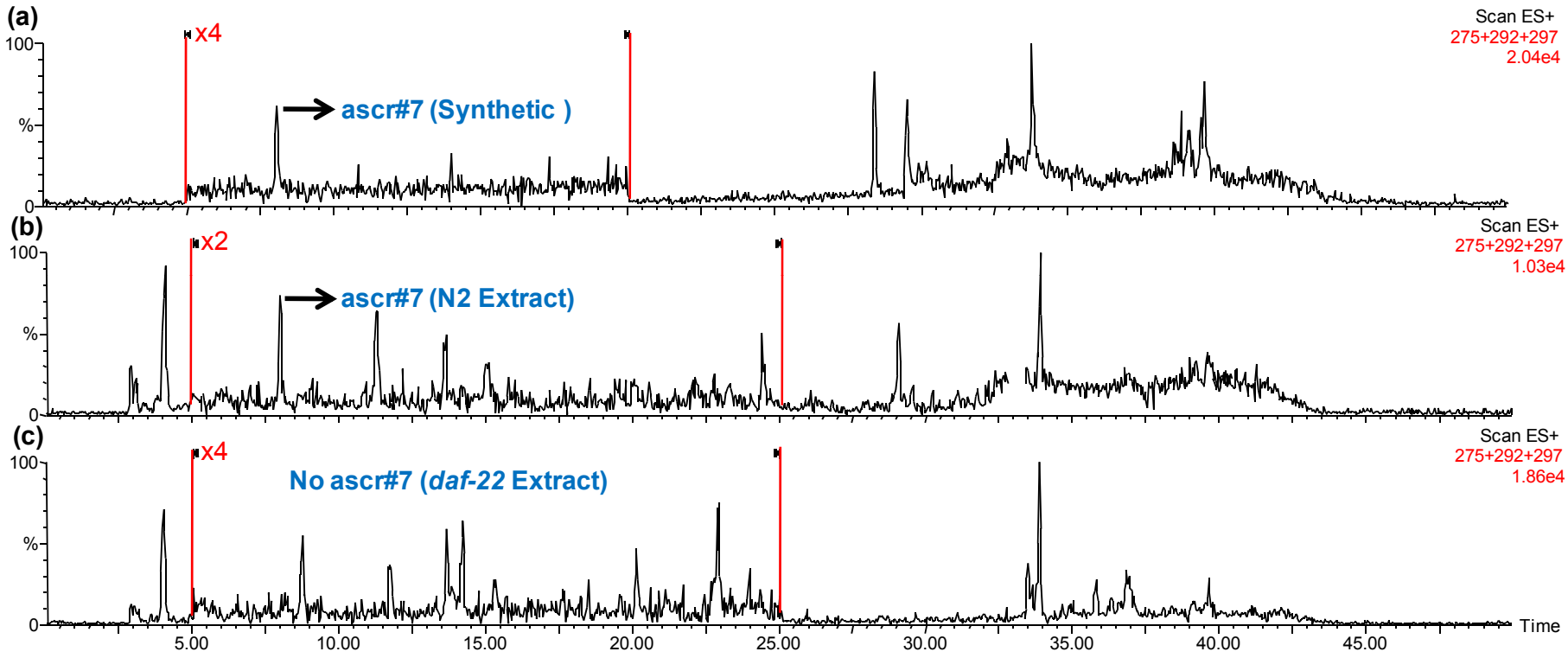
Supporting Figure 12. HPLC-MS analysis of ascr#2. Combined ion chromatograms corresponding to $[M+H]^+$ + $[M+NH_4]^+$ + $[M+Na]^+$ are shown. (**a**) HPLC-MS chromatogram obtained for 45 pmol of ascr#2. (**b**) HPLC-MS chromatogram indicating presence of ascr#2 in wild-type metabolite extract. (**c**) HPLC-MS chromatogram indicating absence of ascr#2 in *daf-22* metabolite extract.



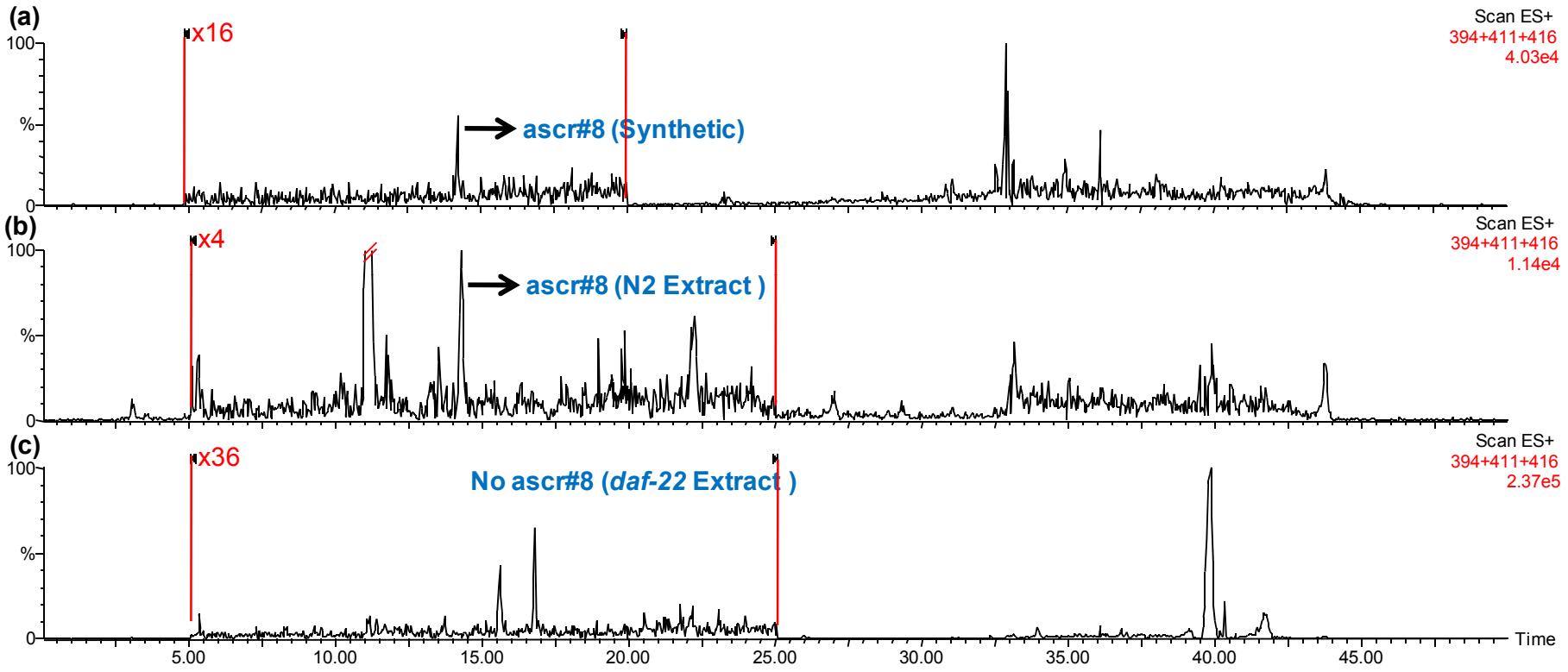
Supporting Figure 13. HPLC-MS analysis of ascr#3. Combined ion chromatograms corresponding to $[M+H]^+$ + $[M+NH_4]^+$ + $[M+Na]^+$ are shown. (**a**) HPLC-MS chromatogram obtained for 45 pmol of ascr#3. (**b**) HPLC-MS chromatogram indicating presence of ascr#3 in wild-type metabolite extract. (**c**) HPLC-MS chromatogram indicating absence of ascr#3 in *daf-22* metabolite extract.



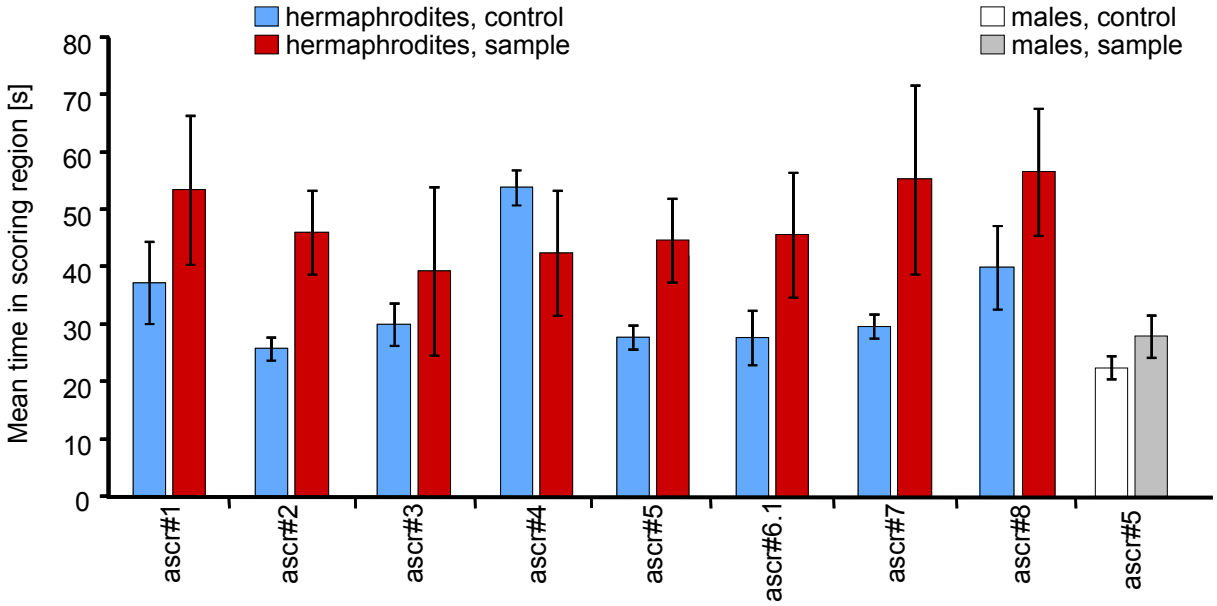
Supporting Figure 14. HPLC-MS analysis of ascr#6.1. Combined ion chromatograms corresponding to $[M+H]^+$ + $[M+NH_4]^+$ + $[M+Na]^+$ are shown. (**a**) HPLC-MS chromatogram obtained for 0.45 nmol of ascr#6.1. (**b**) HPLC-MS chromatogram indicating presence of ascr#6.1 in wild-type metabolite extract. (**c**) HPLC-MS chromatogram indicating absence of ascr#6.1 in *daf-22* metabolite extract.



Supporting Figure 15. HPLC-MS analysis of ascr#7. Combined ion chromatograms corresponding to $[M+H]^+$ + $[M+NH_4]^+$ + $[M+Na]^+$ are shown. (**a**) HPLC-MS chromatogram obtained for 45 pmol of ascr#7. (**b**) HPLC-MS chromatogram indicating presence of ascr#7 in wild-type metabolite extract. (**c**) HPLC-MS chromatogram indicating absence of ascr#7 in *daf-22* metabolite extract.



Supporting Figure 16. HPLC-MS analysis of ascr#8. Combined ion chromatograms corresponding to $[M+H]^+$ + $[M+NH_4]^+$ + $[M+Na]^+$ are shown. (**a**) HPLC-MS chromatogram obtained for 45 pmol of ascr#8. (**b**) HPLC-MS chromatogram indicating presence of ascr#8 in wild-type metabolite extract. (**c**) HPLC-MS chromatogram indicating absence of ascr#8 in *daf-22* metabolite extract.



Supporting Figure 17. Hermaphrodites are not attracted to any of the ascarosides ascr#1-8, and males are not attracted to ascr#5. All compounds were assayed in amounts of 1 pmol. Data for ascr#1-4 has been published previously[8].